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(54) Title: PROTEIN ENGINEERING OF GLUCOAMYLASE TO INCREASE pH OPTIMUM, SUBSTRATE SPECIFICITY AND THERMOSTABILITY

(57) Abstract

A fungal glucoamylase including a mutation pair Asn20Cys coupled with Ala27Cys forming a disulfide bond between the two members of the pair. The mutation provides increased thermal stability and reduced isomaltose formation to the enzyme. A fungal glucoamylase including a 311-314Loop mutation, wherein reduced isomaltose formation is provided by the mutation, is also provided. A fungal glucoamylase, including a mutation Ser411Ala wherein increased pH optimum and reduced isomaltose formation is provided by the mutation, is also provided. Combinations of the mutations in engineered glucoamylases are also provided as are combinations with other glucoamylase mutations that provide increased thermal stability, increased pH optimum and reduced isomaltose formation for cumulative improvements in the engineered glucoamylases.

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**PROTEIN ENGINEERING OF GLUCOAMYLASE TO INCREASE
pH OPTIMUM, SUBSTRATE SPECIFICITY AND THERMOSTABILITY**

FIELD OF THE INVENTION

5 The field of the invention relates to mutations to produce a fungal glucoamylase enzyme that is more selective for the production of glucose rather than the α -1,6 linked disaccharide isomaltose, is more thermostable, and has increased pH optimum and produces 10 increased amounts of glucose compared to wildtype enzymes.

BACKGROUND OF THE INVENTION

15 Glucoamylase (EC 3.2.1.3) is a carbohydrolase. Discovered in 1951, it is an exo-hydrolase that cleaves D-glucose from the nonreducing ends of maltooligosaccharides, attacking α -(1,4)-, and at a much slower rate, α -(1,6)-glucosidic bonds. It is one of more than one hundred carbohydrolases (EC 3.2.1) that 20 cleave O-glycosidic bonds of either α - or β - configuration. The functional and structural relatedness of these enzymes is reflected in the presence of at least three discrete regions of sequence homology between glucoamylase and several α -amylases, α - 25 glucosidases, and transglucanosylases [Svensson, 1988], and a similar domain structure to carbohydrolases that attack insoluble substrates [Knowles et al., 1987; Svensson et al., 1989)]. *Aspergillus awamori* glucoamylase (1,4- α -D-glucan glucohydrolase; EC 30 3.2.1.3) is one of the most important of the glucoamylases.

35 Glucoamylase is primarily used in industry for the production of high-fructose corn sweeteners in a process that involves 1) α -amylase to hydrolyze starch to maltooligosaccharides of moderate length (dextrin); 2) Glucoamylase to hydrolyze dextrin to glucose; and 3)

glucose isomerase to convert glucose to fructose. Corn sweeteners have captured over 50% of the U. S. sweetener market, and the three enzymes used to make them are among the enzymes made in highest volume. In 5 addition, glucose produced by glucoamylase can be crystallized or used in fermentation to produce organic products such as citric acid, ascorbic acid, lysine, glutamic acid or ethanol for beverages and fuel. Approximately 12% of the country's corn production is 10 processed with glucoamylase. Although glucoamylase has been successfully used for many years, it would be a more attractive product if it produced higher amounts of glucose instead of disaccharides, if it were more stable, and if it could be used in the same vessel with 15 glucose isomerase.

Glucoamylase does not give 100% yield of glucose from dextrin because it makes various di- and trisaccharides, especially isomaltose and isomaltotriose, from glucose [Nikolov et al., 1989]. 20 These products, formed at high substrate concentrations, result from the ability of glucoamylase to form α -(1,6)-glucosidic bonds. Glucoamylase is not as thermostable as either α -amylase or glucose isomerase. The optimum pH of GA (pH4-4.5) is lower 25 than that of α -amylase (pH5.5-6.5) and glucose isomerase (pH7-8). Therefore glucoamylase hydrolysis must be done separately from the other enzymatic reactions in a different vessel and at lower temperatures, causing higher capital costs.

30 Glucoamylase from the filamentous fungus *Aspergillus niger* is the most widely used glucoamylase, and its biochemical properties have been extensively characterized. This enzyme is found mainly in two forms, GAI (616 amino acids; referred to as AA 35 hereinafter) and GAI (512 AA), differing by the presence in GAI of a 104-AA C-terminal domain required for

adsorption to native starch granules [Svensson et al., 1982; Svensson et al., 1989]. Both forms have a catalytic domain (AA1-440) followed by a Ser/Thr-rich, highly O-glycosylated region (AA441-512) [Gunnarsson et al., 1984]. The first thirty residues of this region are included in the three-dimensional structure of the enzyme [Aleshin et al., 1994; 1996; Stoffer et al., 1995]; they wrap around the catalytic domain like a belt. There is strong AA sequence homology among 5 fungal glucoamylase's in four distinct regions of the catalytic domain that correspond to the loops that form the substrate binding site [Itoh et al., 1987]. In *A. niger* glucoamylase these regions are AA35-59, AA104-134, AA162-196, and AA300-320. The second and third 10 regions partially or completely overlap the three regions of homology to α -amylases [Svensson, 1988]. In addition, the raw starch binding domain (AA512-616) has high homology to similar domains from several starch-degrading enzymes [Svensson et al., 1989].

20 Kinetic analysis showed that the substrate binding site is composed of up to seven subsites [Savel'ev et al., 1982] with hydrolysis occurring between subsites 1 and 2. The pK_s 's of hydrolysis, 2.75 and 5.55 [Savel'ev and Firsov, 1982], suggest that carboxylic acid 25 residues at subsites 1 and 2 provide the catalytic acid and base for hydrolysis. Chemical modification experiments showed that three highly conserved residues, Asp176, Glu179, and Glu180, are protected and are in the active site, suggesting that one or more of 30 them are the possible catalytic residues [Svensson et al., 1990]. Chemical modification experiments also indicated that the highly conserved residue Trp120 is essential, and is located in subsite 4 [Clarke and Svensson, 1984]. Trp120 is homologous to Trp83 of 35 *Aspergillus oryzae* α -amylase [Clarke and Svensson, 1984], which is also located in the active site of that

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enzyme [Matsuura et al., 1984]. Site directed mutagenesis studies have indicated that Glu179 is the catalytic acid residue, while Glu400 is the catalytic base residue [Frandsen et al., 1994; Harris et al., 1993; 5 Sierks et al., 1990]

Glucoamylases from *A. niger* [Svensson et al., 1983; Boel et al., 1984] and *Aspergillus awamori* [Nunberg et al., 1984] have been cloned and sequenced, and have identical primary structures. Innis et al. 10 [1985] and more recently Cole et al. [1988] have developed vectors (pGAC9 and pPM18, respectively) for glucoamylase expression in yeast, allowing convenient manipulation and testing of glucoamylase mutants.

15 **SUMMARY OF THE INVENTION**

According to the present invention, a fungal glucoamylase (1,4- α -D-glucan glucohydrolase; EC 3.2.1) with decreased thermal inactivation (increased 20 thermostability) and reduced isomaltose formation provided by the mutation Asn20Cys coupled with Ala27Cys forming a disulfide bond between the two is provided. Cumulative thermostability is also provided for GA by including the mutation Asn20Cys coupled with Ala27Cys 25 and at least one mutation from Table 13. An engineered GA including Ser30Pro, Gly137Ala, and Asn20Cys coupled with Ala27Cys provides even more thermostability. Cumulative thermostability is also provided for GA by including the mutation Asn20Cys 30 coupled with Ala27Cys and at least two mutations from Table 13.

The present invention also provides a fungal glucoamylase with reduced isomaltose formation including an Asn20Cys coupled with Ala27Cys mutation 35 (S-S mutation) and at least one mutation selected from Table 14. In an embodiment Asn20Cys coupled with

Ala27Cys mutation and a 311-314Loop (also referred to as 300Loop) mutation are included in an engineered GA.

In a further preferred embodiment the engineered glucoamylase with reduced isomaltose formation includes

5 Asn20Cys coupled with Ala27Cys mutations Ser30Pro and Gly137Ala.

The present invention also provides engineered fungal glucoamylase including a 311-314Loop mutation whereby reduced isomaltose formation is provided by the 10 mutation. In a further embodiment fungal glucoamylase including a 311-314Loop mutation and at least one mutation from Table 14 are prepared whereby cumulative reduced isomaltose formation is provided by the additional mutation.

15 The present invention provides a fungal glucoamylase including a mutation Ser411Ala whereby increased pH optimum and reduced isomaltose formation is provided by the mutation. In an embodiment the Ser411Ala mutation is combined with at least one 20 mutation from Table 15 whereby cumulative increased pH optimum is provided by the mutations. In an embodiment the Ser411Ala mutation is combined with at least one mutation from Table 14 whereby cumulative reduced isomaltose formation is provided by the mutations.

25 In a further embodiment an engineered fungal glucoamylase includes a mutation Ser411Ala and a mutation pair Asn20Cys coupled with Ala27Cys forming a disulfide bond between the two members of the pair whereby increased thermal stability, increased pH 30 optimum and reduced isomaltose formation are provided by the mutations.

In a still further embodiment a fungal glucoamylase is engineered to include a Ser411Ala mutation and a mutation pair Asn20Cys coupled with 35 Ala27Cys forming a disulfide bond between the two members of the pair and a 311-314Loop mutation whereby

increased thermal stability, increased pH optimum and reduced isomaltose formation are provided by the mutations.

The present invention provides a method to obtain 5 a fungal glucoamylase with reduced isomaltose formation by designing mutations to decrease the α -(1,6)- glucosidic linkage affinity of GA.

The present invention also provides a method to obtain a fungal glucoamylase with decreased thermal 10 inactivation by designing mutations to decrease the enzyme's conformational entropy of unfolding and/or increase stability of α -helices, increase disulfide bonds, hydrogen bonding, electrostatic interactions, hydrophobic interactions, Vanderwalls interactions and 15 packing compactness.

The present invention also provides a fungal glucoamylase with increased pH optimum including changing the polarity, charge distribution and hydrogen bonding in the microenvironment of the catalytic base 20 Glu400.

The present invention also provides a method of genetically engineering glucoamylase carrying at least two cumulatively additive mutations. Individual mutations are generated by site-directed mutagenesis. 25 These individual mutations are screened and those selected which show increased pH optimum and which show decreased irreversible thermal inactivation rates or reduced isomaltose formation. Site directed mutagenesis is then performed to produce enzymes 30 carrying at least two of the isolated selected mutations. Finally the engineered enzymes are screened for cumulatively additive effects of the mutations on thermal stabilizing or reduced isomaltose formation by the produced enzymes carrying at least two of the 35 isolated selected mutations. Alternatively, the engineered enzyme is screened for cumulatively additive

effects of both of the mutations on pH optimum, thermostability and/or reduced isomaltose formation by the produced enzymes carrying at least two of the isolated selected mutations.

5 Vectors for each of the mutations and mutation combinations are also provided by the present invention as well as host cells transformed by the vectors.

DESCRIPTION OF THE DRAWINGS

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Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the 15 accompanying drawings wherein:

FIGURE 1 is a graph showing the relationship between temperature and k_d for wild-type (●) and proline substituted mutant GA's: S30P (■), D345P (▽), E408P (◇) in Example 1.

20

FIGURE 2 is a graph showing effect of temperature on first-order thermoinactivation rate coefficients of wild-type (○), A27C (●), N20C (▽), A27C/N20C (▼), A471C/T72C (□), A27C/N20C/G137A (■), A27C/N20C/S436P (◇) AND G137A/S436P (◆) glucoamylases measured in pH 25 4.5 buffer.

25

FIGURE 3 is a graph showing initial reaction rates of wild-type (○), A27C/N20C (●), A471C/T72C (▽) and A29C/N20C/G137A (▼) glucoamylases with 4% maltose in 0.05 M sodium acetate (pH 4.5) as substrate at 30 temperatures from 60°C to 76°C.

30

FIGURE 4 is a graph showing the effect of temperature on the activity of wildtype and mutant GA. Error bars represent the standard deviation from three assays. Wildtype (●), S30P/G137A (□), S-S/S30P/G137A (▲).

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FIGURE 5A-C are graphs showing the effect of

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temperature on irreversible thermal inactivation rate coefficients of wildtype and mutant GA. Fig. 5A Wildtype (●), S30P (■), G137A (△), S30P/G137A (□); Fig. 5B Wildtype (●), S30P (■), S-S (hexagon), S-S/S30P (filled circle with empty center); Fig. 5C Wildtype (●), S30P/G137A (□), S-S/S30P (filled circle with empty center), S-S/S30P/G137A (△).

FIGURE 6A-B are graphs showing saccharification of 28% (w/v) Maltrin M100 by wildtype (●), S30P/G137A (□) and S-S/S30P/G137A (△).

FIGURE 7 is a graph showing the 30% DE 10 maltodextrin saccharification of wildtype (♦) and mutant glycoamylases: 300Loop (■), S30P/G137A (△), S-S (●), S30P/G137A/300Loop (×), S-S/300Loop (△), at 55°C, enzyme concentration was 166.67 µg/mL in each reaction.

FIGURE 8 is a graph showing production of isomaltose by wildtype (●) and mutant glucoamylases: Y116W (■), Y175F (△), R241K (▼), S411A (♦), S411G (hexagon), during glucose condensation at 55°C with 30% (w/v) D-glucose in 0.05M sodium acetate buffer at pH4.4 with 0.02% sodium azide for 12 days.

FIGURE 9 is a graph showing the production of glucose by wildtype (●) and mutant glucoamylases: Y116W (■), Y175F (△), R241K (▼), S411A (♦), S411G (hexagon), during hydrolysis of DE 10 maltodextrin at 55°C with 28% (w/v) maltodextrin in 0.05M sodium acetate buffer at pH4.4 with 0.02% sodium azide for 12 days.

FIGURE 10 is a graph showing the initial rates of glucose production by wildtype (●) and S411A (■) glucoamylases during DE 10 maltodextrin hydrolysis at different pH values. Hydrolysis was performed at 36°C with 28% (w/v) maltodextrin in 25mM citrate-phosphate buffer at indicated pHs with 0.02% sodium azide for 4 days.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides mutations for increased thermal stability, increased pH optimum and reduced isomaltose formation in the glucoamylase from fungal species which may provide increased glucose yields compared to wildtype glucoamylase. Predicted structure and known sequences of glucoamylase are conserved among the fungal species [Coutino et al, 1994]. As an exemplar *Aspergillus awamori* glucoamylase (1,4- α -D-glucan glucohydrolase; EC 3.2.1.3; referred to as GA herein; SEQ ID No:1) is used, but any other fungal species including *Aspergillus* species glucoamylase can be used. The numbering of the glucoamylase amino acids herein is based on the sequence of the exemplar *Aspergillus awamori*. Equivalent amino acid residue numbers are determined differently for different fungal species as is known in the art [Coutino et al., 1994].

The present invention provides a fungal glucoamylase with decreased thermal inactivation (increased thermostability) and decreased isomaltose formation provided by engineering the inclusion of a mutation pair Asn20Cys coupled with Ala27Cys which forms a disulfide bond between them (this mutation is abbreviated as Asn20Cys/Ala27Cys or S-S). Additional mutations providing decreased thermal inactivation are set forth in Summary Table 13.

Cumulative thermostability is also provided for GA by including at least two of the mutations in the enzyme as for example including mutations Ser30Pro and Gly137Ala. Another example is to engineer S-S with Asn20Cys/Ala27Cys in the enzyme or to pair Gly137Ala with S-S. Further, combinations of the individual mutations set forth in Table 13, particularly with S-S coupled with Ser30Pro also provide cumulative

thermostability. In general two mutation combinations are made but triple mutations can also be constructed. As for example, an engineered GA including the three mutations: Ser30Pro, Gly137Ala, and Asn20Cys/Ala27Cys 5 provides even more thermostability.

By Asn20Cys coupled with Ala27Cys is meant a pair of mutations which is abbreviated as "S-S" or Asn20Cys/Ala27Cys and between which is formed a disulfide bond as described herein in the Examples. In 10 general, this is referred to as a single mutation since both are required to form the disulfide bond.

By cumulative is generally meant the additive (or nearly additive) effects of two or more mutations on the parameter of enzyme activity being measured.

15 The present invention also provides a fungal glucoamylase with reduced isomaltose formation and increased glucose yield including the Asn20Cys/Ala27Cys mutation (S-S mutation) and at least one mutation selected from Table 14. In an embodiment the 20 Asn20Cys/Ala27Cys mutation and the 311-314Loop (300Loop) are included in GA. In a further preferred embodiment the engineered glucoamylase with reduced isomaltose formation includes Asn20Cys/Ala27Cys and with mutations Ser30Pro and Gly137Ala.

25 In an embodiment a glucoamylase with the 311-114 loop mutation is constructed to provide reduced isomaltose formation. By the 311-314Loop mutation is meant an insertional GA mutant with the sequence Tyr311-Tyr312-Asn313-Gly314→Tyr311-Asn-Gly-Asn-Gly-Asn- 30 Ser-Gln-Gly314 (311-314 Loop; SEQ ID No:2).

35 The present invention provides a fungal glucoamylase including a Ser411Ala mutation whereby increased pH optimum and reduced isomaltose formation is provided by the mutation. In an embodiment the Ser411Ala mutation is combined with at least one mutation from Table 15 whereby cumulative increased pH

optimum is provided by the combined mutations. In a further embodiment the Ser411Ala mutation is combined with at least one mutation from Table 14 whereby cumulative reduced isomaltose formation is provided by 5 the mutations.

In a further embodiment an engineered fungal glucoamylase includes a Ser411Ala mutation and the mutation pair Asn20Cys/Ala27Cys forming a disulfide bond between them whereby increased thermal stability, 10 increased pH optimum and reduced isomaltose formation are provided by the mutations.

In a still further embodiment a fungal glucoamylase including a Ser411Ala mutation and a mutation pair Asn20Cys coupled with Ala27Cys forming a disulfide bond between the two members of the pair and a 311-314Loop mutation whereby increased thermal 15 stability, increased pH optimum and reduced isomaltose formation are provided by the combination of mutations.

Mutations are indicated by the amino acid being 20 replaced followed by the residue number followed by the replacing amino acid. Amino acids are abbreviated either with the three letter code or single letter code. Mutations are generated using site directed 25 mutagenesis as is known in the art. The sequence and residue number are from the Wildtype (WT) or nonmutant enzyme. Biochemical characterization is performed as described herein below and in the Examples. The Examples provide exemplars of the analysis for an individual mutation to determine it's characteristics 30 and provide exemplars of analysis for combinations of mutations to determine if the combination provides a cumulative effects.

By increased thermostability (or decreased thermal inactivation) is meant that at temperatures between 35 65°C and 77.5°C the mutants are irreversibly inactivated at a decreased rate compared to wildtype.

The present invention provides a method to obtain fungal glucoamylases with decreased thermal inactivation by designing mutations to decrease the rate of irreversible thermal inactivation at 5 temperatures between 65°C and 77.5°C compared to wildtype. This is accomplished by designing glucoamylases with decreased thermal inactivation by designing mutations to decrease the enzyme's conformational entropy of unfolding and/or increase 10 stability of α -helices, increase disulfide bonds, hydrogen bonding, electrostatic interactions, hydrophobic interactions, Vanderwalls interactions and packing compactness.

Basic mechanisms underlying protein

15 thermostability and factors influencing reversible and irreversible thermal inactivation have been studied extensively [Argos et al., 1979; Klibanov, 1983; Wasserman, 1984; Ahern and Klibanov, 1985]. Factors involved in stabilizing proteins at high temperatures 20 include 1) disulfide bonds, 2) noncovalent bonds such as salt bridges, hydrogen bonding, and hydrophobic interactions, and 3) conformational rigidity [Nosoh and Sekiguchi, 1988]. The causes of irreversible inactivation at high temperatures include 1) 25 aggregation, 2) the formation of incorrect structures, 3) the destruction of disulfide bonds, 4) deamidation (especially of Asn at Asn-Gly sequences), and 5) cleavage of Asp-X peptide linkages. It is apparent that replacement of even one residue can make a large 30 difference in protein thermostability [Matsumura and Aiba, 1985], due to the small increases in free energy (20-30 kJ/mol) usually required to stabilize protein tertiary structures [Nosoh and Sekiguchi, 1988]. Genetic engineering to increase thermostability (or to 35 decrease irreversible thermoinactivation) of enzymes has been successful in several cases [Perry and Wetzel,

1984; Imanaka et al., 1986; Ahearn et al., 1987]. However, the mechanisms that govern thermostability are not fully understood, so that amino acid (AA) replacements that promote thermostability are not 5 accurately predicted [Leatherbarrow and Fersht, 1986; Nosoh and Sekiguchi, 1988; Pakula and Sauer, 1989]. The method of the present invention allows for more accurate prediction.

By increased pH optimum is meant that the enzyme 10 is functional at a higher pH, above that of wildtype.

The present invention also provides a method to design a fungal glucoamylase with increased pH optimum by changing the polarity, charge distribution and hydrogen bonding in the microenvironment of the 15 catalytic base Glu400. For example, mutants S411G and S411A were designed to remove the hydrogen bond between Ser411 and Glu400 (see Example 8).

By increased selectivity is meant that there is decreased isomaltose formation due to decrease in the 20 production of undesirable α -(1,6)-linked byproducts (reversion products) at high glucose concentrations [Lee et al., 1976]. As described above, GA hydrolyzes and synthesizes both α -(1,4) and α -(1,6) glucosidic bonds. Increasing selectivity indicates that the 25 enzyme synthesizes α 1,6 linked products at a lower rate than wildtype as shown by reduced levels of isomaltose formation in condensation reactions with 30% glucose as a substrate compared to wildtype GA. Additionally, improved selectivity may result in increased glucose 30 yields in saccharification reactions using 28% DE 10 maltodextran as a substrate.

The present invention provides a method to obtain a fungal glucoamylase with reduced isomaltose formation by designing mutations to decrease the α -(1,6)- 35 glucosidic linkage affinity of GA. That is mutations are designed in the active site to reduce isomaltose

formation due to glucose condensation. The mutations are designed to have decreased ability to synthesize isomaltose while maintaining at least partial wildtype ability to digest α 1-4 linked substrates resulting in a 5 lower ratio of isomaltose formation to glucose formation than wildtype. These mutations are made at positions that are not completely conserved based on homology analysis.

Kinetic studies have indicated that there are five 10 to seven glucosyl binding subsites, and the catalytic site is located between subsites 1 and 2 [Hiromi et al., 1973, Hiromi et al., 1983, Meagher et al., 1989, Tanaka et al., 1983]. The solved three-dimensional 15 structure of the catalytic domain of glucoamylase from *Aspergillus awamori* var X100, which has about 95% homology with the corresponding regions of GAs from *Aspergillus awamori* and *Aspergillus niger* [Coutinho & Reilly, 1994], contains thirteen alpha-helices, twelve 20 of which are arranged in pairs forming an alpha/alpha barrel [Aleshin et al., 1992, Aleshin et al., 1994]. The active site is located in the cavity of the barrel center. In addition, homology analysis of thirteen 25 amino acid sequences of glucoamylases showed that five conserved regions define the active site [Coutinho & Reilly, 1994]. The mechanism of GA catalysis involves two carboxyl groups [Hiromi et al., 1966], Glu179 and Glu400 (in *Aspergillus awamori* or *Aspergillus niger*) [Frandsen et al., 1994, Harris et al., 1993, Sierks et al., 1990]. Glu179 protonates the oxygen in the 30 glycosidic linkage, acting as general acid acatalyst, and Glu400 activates water (Wat500) for nucleophilic attack at carbon C-1, acting as a general base catalyst [Frandsen et al., 1994]. The crystal structures of glucoamylase complexed with the pseudotetrasaccharides 35 (acarbose and D-gluco-dihydroacarbose), showed that there are two different binding conformers, pH 4-type

and pH 6-type, for pseudotetrasaccharides at pH 4 [Aleshin et al., 1996, Stoffer et al., 1995]. Binding of the first two sugar residues of the pseudotetrasaccharides is the same, but there is an 5 extraordinary variation in binding of the third and fourth sugar residues of the pseudotetrasaccharides [Stoffer et al., 1995].

The substrate specificity of an enzyme is determined by its ability to form a stable complex with 10 a ligand in both the ground state and the transition state. The stability of the enzyme-ligand complex is affected by steric constraints, hydrogen bonding, van der Waal's and electrostatic forces, and hydrophobic contacts [see generally Fersht, 1985 *Enzyme Structure and Mechanism*, 2nd edition, Freeman, San Francisco]. 15 Site-directed mutagenesis was used to construct mutations at residues 119 and 121 to alter the hydrogen bonding between enzyme and substrate. Atom OG of Ser119 hydrogen bonds to the 3-OH of the fourth sugar 20 residue of pseudo-tetrasaccharides only in the pH 6-type conformer, whereas the amide nitrogen of Gly121 hydrogen bonds to the 6-OH of the third sugar residue in both pH 4-type and pH 6-type conformers. These 25 mutations are designed to change substrate specificity (decrease alpha-1,6 condensation reactions) while maintaining wild-type ability to hydrolyze alpha-1,4 linked substrates. Ser119 is not conserved and is replaced by Ala, Pro and Glu in other GAs. Mutant S119E was designed to strengthen the hydrogen bond 30 between the enzyme and the fourth sugar residue of the substrate to stabilize the pH 6-type conformer, and to bring a negative charge near subsite 4 in order to increase electrostatic interactions in active site. Mutant S119G was designed to remove the same hydrogen 35 bond in order to destabilize the pH 6-type conformer. Mutant S119W was designed to remove the same hydrogen

bond and to increase the hydrophobic interactions between the enzyme and the pH 6-type conformer. Gly121 is highly conserved in all glucoamylase sequences except in *Clostridium sp.* G005 GA, which has high α -1,6 activity and in which Gly is replaced by Thr. Since the ϕ and ψ angles of Gly121 would allow an alanine in this position without causing a conformation distortion, G121A was designed to introduce a Beta-carbon at position 121 to displace the 6-OH group of the third sugar residue from its hydrogen bonding position. In addition, the double mutant G121A/S411G was designed to investigate additivity of the two substrate specificity mutations. S411G is shown herein to reduce the ratio of initial rates of isomaltose production (from glucose condensation reactions) to that of glucose production (from the hydrolysis of DE 10 maltodextrin).

The following provide further examples of the strategies used for the design of mutations having increased selectivity.

300Loop mutation: According to the amino acid sequence homology study [Countinho and Reilly, 1994], it was found that GAs from *Rhizopus* and some other fungal families have a longer amino acid sequence and form a larger loop or cavity in the S4 conserved region compared to *A. niger* or *A. awamori* GAs. Since single mutation events alone are unlikely to bring about substantial increase in the specificity of bond hydrolysis or synthesis, an insertional mutant GA was designed, designated 300Loop or 311-314Loop (SEQ ID NO:2), and the inserted seven amino acids were adapted from *Rhizopus* GA because *Rhizopus* GA was the first enzyme to which the subsite theory was successfully applied [Himori et al., 1973]. The 300Loop mutation was designed to decrease the α -(1,6)-glucosidic affinity by introducing a larger loop into the S4

conserved region.

Tyr175Phe: Tyr175 is within the third conserved region.

The nearest distance between Tyr175 and the fourth residue of inhibitor α -gluco-dihydroacarbose is 4.06 Å

5 [Stoffer et al., 1995]. Tyr175 is replaced by Phe or Gln in several other glucoamylases. Changing Tyr175 to Phe was designed to increase the hydrophobic interaction between enzyme and substrate.

Gly121Ala: Gly121 is highly conserved in all

10 glucoamylase sequences except in *Clostridium* sp. G005 GA, which has high α -1,6 activity and in which Gly is replaced by Thr. Since the ϕ and ψ of Gly121 would allow an alanine in this position without causing a conformation distortion, G121A was designed to

15 introduce a β -carbon at position 121 to displace the 6-OH group of the third sugar residue from its hydrogen bonding position.

Gly121Ala with S411G (generally indicated as

G121A/S411G): The double mutant was designed to

20 investigate additivity (cumulative) of the two substrate specificity mutations. S411G reduces the ratio of initial rates of isomaltose production (from glucose condensation reactions, see Examples) to that of glucose production (from the hydrolysis of

25 maltodextrin 10).

The present invention provides a method of engineering mutations for fungal glucoamylase and then preparing engineered enzymes carrying cumulatively additive mutations. The initial step is to generate 30 individual mutations by site directed mutagenesis and screen the individual mutations as described in the Examples. Those individual mutations which show decreased irreversible thermal inactivation rates or reduced isomaltose formation or increased pH optimum 35 are then selected for combinatorial analysis. In general mutations are selected which have at least

wildtype reaction rates.

Mutations are combined by site-directed mutagenesis to determine if their effects are additive as is discussed herein in the Examples. Site directed 5 mutagenesis to produce enzymes carrying at least two of the isolated selected mutations is performed as is known in the art. These engineered enzymes are then screened for cumulatively additive effects on thermal stabilizing, pH optimum or reduced isomaltose 10 formation. Alternatively the engineered enzymes carrying cumulative mutations are screened for cumulative effects on two or more of the parameters.

For biochemical characterization of the mutants, GA is purified from culture supernatants of 15-L batch 15 fermentations by ultrafiltration, DEAE-Sephadex column chromatography, and column affinity chromatography using the potent inhibitor acarbose attached to a support [Sierks et al., 1989]. Purities of the resulting preparations are tested by standard 20 techniques such as SDS-polyacrylamide gel electrophoresis and isoelectric focusing with narrow-band ampholytes. Protein are measured by absorbance at 280 nm or by Bradford's method [1976]. GA activity is measured by a glucose oxidase/o-dianisidine assay 25 (Sigma).

Selectivity is determined by any method known in the art but preferably by measuring the initial rate of isomaltose formation from 30% (w/v) glucose condensation reactions at pH 4.4 and 55°C in 0.05M 30 sodium acetate buffer and then by measuring the initial rate of glucose formation in 30% (w/v) DE 10 maltodextran hydrolysis reactions at pH 4.4 and 55°C 0.05M sodium acetate buffer. From the resulting initial rates, the ratio of isomaltose formation to 35 glucose formation is calculated.

Thermostability is measured as is known in the art

but preferably by incubating the enzyme at selected temperatures between 65°C and 77.5°C at 2.5°C intervals followed by activity analysis at 35°C using 4% maltose as substrate. When first-order decay is observed, as 5 with WT GA, decay rate coefficients are determined. Activation energies for decay are calculated from the rate coefficients at different temperatures.

pH optimum is measured as is known in the art but preferably at 45°C at 16 pH values, ranging for 2.2 to 10 7.0 using 0.025 M citrate-phosphate buffer with maltose or maltoheptaose as substrate.

Saccharification is measured as described in the Examples. Briefly, glucoamylase is incubated with DE 10 maltodextran as substrate in 0.05M sodium acetate 15 buffer at pH 4.4 at 55°C. Samples are taken at various times from 0.5 to 288 hours and the production of glucose determined.

The present invention provides vectors comprising an expression control sequence operatively 20 linked to the nucleic acid sequence of the various mutant sequences disclosed herein, combinations of mutations and portions thereof. The present invention further provides host cells, selected from suitable eucaryotic and prokaryotic cells, which are transformed 25 with these vectors.

Vectors can be constructed containing the cDNA of the present invention by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the sequences. 30 Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the nucleic acids in a different form. Examples are provided herein. Phagemids are a specific example of such beneficial vectors because they can be used either 35 as plasmids or as bacteriophage vectors. Examples of other vectors include viruses such as bacteriophages,

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baculoviruses and retroviruses, DNA viruses, cosmids, plasmids, liposomes and other recombination vectors. The vectors can also contain elements for use in either prokaryotic or eukaryotic host systems. One of 5 ordinary skill in the art will know which host systems are compatible with a particular vector.

The vectors can be introduced into cells or tissues by any one of a variety of known methods within the art (calcium phosphate transfection; 10 electroporation; lipofection; protoplast fusion; polybrene transfection; ballistic DNA delivery; lithium acetate or CaCl transformation). The host cell can be any eukaryotic and prokaryotic cells, which can be transformed with the vector and which will support the 15 production of the enzyme.

The above discussion provides a factual basis for thermostable and selective mutants of fungal glucoamylase as well as methods of designing the mutations and screening for the cumulative effect of 20 the mutations and vectors containing the mutations. The methods used with and the utility of the present invention can be shown by the following non-limiting examples and accompanying figures.

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EXAMPLES

General methods in molecular biology: Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989) and Rose, et al. *Methods in Yeast Genetics: A Laboratory Course Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1990). 30 35 Polymerase chain reaction (PCR) was carried out

generally as in *PCR Protocols: A Guide To Methods And Applications*, Academic Press, San Diego, CA (1990). Oligonucleotides are synthesized as is known in the art. For example, an Applied Biosystems 380B DNA synthesizer can be used.

Materials: *S. cerevisiae* C468 (α leu2-3 leu 2-112 his 3-11 his 3-15 mal⁻) and the plasmid YEpPM18 were gifts from Cetus. Acarbose was a gift from Miles Laboratories. All restriction enzymes were purchased from Promega as well as T4 DNA ligase and pGEM-7Z(+), an *E. coli* phagemid vector, were from Promega. Maltose (G₂), maltotriose (G₃), maltotetraose (G₄), malto-pentaose (G₅), maltohexaose (G₆), maltoheptaose (G₇), glucose oxidase, peroxidase, and α -naphthol were from Sigma. Isomaltose (iG₂) was purchased from TCI America. DE 10 Maltodextrin with the average degree of polymerizations (DP) of 10, 6, and 4, respectively, were from Grain Processing Corporation. High-performance thin-layer chromatographic (HPTLC) plates (LHPK silica gel 60 Å, 20 x 10 cm) were from Whatman.

Site-directed mutagenesis: Site-directed mutagenesis was performed according to the Muta-Gene phagemid *in vitro* mutagenesis kit from Bio-Rad which is based on the method of Kunkel et al [1985]. A 1.7 kb XhoI \rightarrow BamHI DNA fragment coding for the glucoamylase catalytic domain was cloned into a pBluescript II KS(+) vector from Stratagene. Oligonucleotides used as mutagenic primers are provided with the specific Example. The presence of the individual mutations was confirmed by sequencing and each mutated GA gene fragment was subcloned into YEpPM18 [Cole, et al., 1988] and transformed into *S. cerevisiae*.

Enzyme production and purification: Wild-type (WT) and mutant enzymes are produced by growing yeast at 30°C in 5.3 L SD + His media for 72 hours at pH 4.5 in a 5.0 L fermentor. After 48 hours, 100g of dextrose

and 22g of $(\text{NH}_4)_2\text{SO}_4$ in 300ml H_2O is added as a supplement. Following growth, the culture is centrifuged to remove yeast cells, the supernatant is concentrated by ultrafiltration, diafiltered against 5 0.5 M NaCl/0.1 M NaOAc, pH 4.5 and purified by acarbose-sepharose affinity chromatography. GA is eluted with 1.7 M Tris-Cl, pH 7.6, dialyzed against H_2O , further concentrated by ultrafiltration and diafiltered 10 against 0.05 M NaOAc buffer, pH 4.5. The protein concentration is determined according to the Pierce 15 bicinchoninic acid protein assay [Smith et al., 1985] using bovine serum albumin as a standard.

Enzyme activity assays: Enzyme activities were determined at 50°C using 4% maltose in 0.05 M NaOAc 20 buffer pH 4.5 as substrate. One international unit (IU) of enzyme activity was defined as the amount of enzyme required to produce 1 $\mu\text{mol}/\text{min}$ glucose at assay conditions. Following mixing enzyme with substrate, six 100 μl samples were removed at seven minute intervals over 42 minutes, the reaction stopped with 40 μl of 4.0 M Tris-Cl, pH 7.0 and the glucose concentration was determined according to the Sigma 25 peroxidase-glucose oxidase/o dianisidine glucose assay kit.

Irreversible thermal inactivation: Duplicate aliquotes of 40 $\mu\text{g}/\text{ml}$ of purified wild-type and mutant enzymes were subjected to inactivation at six or more temperatures between 65° and 80°C at intervals of 30 2.5°C. Samples were removed at six different time points, immediately placed on ice and stored at 4°C for 24 hours. The residual activity of the inactivated samples along with a corresponding sample which had not been subjected to thermal inactivation, was determined as described above but at 35°C.

pH dependence of glucoamylase activity: pH dependence of glucoamylase activity was measured at

45°C at 16 different pH values, ranging from 2.2 to 7.0, using 0.025 M citrate-phosphate buffer [McIlvane, 1921] with maltose or maltoheptaose as substrate. The 5 ionic strength of the citrate-phosphate buffer was maintained at 0.1 by adding potassium chloride. The pH values of free enzyme and enzyme-substrate complex were measured at substrate concentrations (i) smaller than 0.2 K_m , so that the initial rate (v) was proportional to k_{cat}/K_m , and (ii) higher than 10 K_m , so that the initial 10 rate (v) was proportional to k_{cat} [Sierks & Svensson, 1994, see also Whitaker (1994) Principle of enzymology for the food sciences, 2nd edition, Marcel Dekker, NY]. The pH values of two catalytic groups of free enzyme and enzyme-substrate complex were calculated by fitting 15 the initial rates as a function of pH values to the equation $\log Y = \log [C/(1+H/K_1+K_2/H)]$ by using the software of Enzfitter. Y is the observed value of the parameter of interest (i.e. k_{cat}/K_m or k_{cat}) measured at different pH values, C is the pH independent value of Y 20 (i.e. the maximal value of k_{cat}/K_m or k_{cat}), H is the concentration of hydrogen ion, K_1 and K_2 are dissociation constants of catalytic groups of enzyme. When the values of apparent pK_1 and pK_2 were separated by less than 3 pH units, the pH values were adjusted by 25 equations $(H^*)_1 + (H^*)_2 = K_1 + 4(H^*)_{opt}$ and $(H^*)_{opt} = \sqrt{K_1 K_2}$ [Whitaker, 1994]. The concentration of hydrogen ion at the optimum pH, $(H^*)_{opt}$, was calculated from pH_{opt} which is equal to the average of apparent pK_1 and pK_2 . $(H^*)_1$ and $(H^*)_2$ (apparent K_1 and K_2) correspond to the 30 concentrations of hydrogen ion when the pH values are equal to apparent pK_1 and pK_2 , respectively.

The hydrolysis of DE 10 maltodextrin

(Saccharification): Hydrolysis was performed at 35°C and/or 55°C (as indicated in the text) with 28% (w/v) 35 DE 10 maltodextrin as substrate in 0.05 M sodium acetate buffer at pH 4.4 with the addition of 0.02%

sodium azide, used to inhibit microbial growth in the reaction mixtures. The enzyme concentration was 2.64 μ M for both wild-type and mutant GAs. Samples were taken at various times (from 0.5 to 288 hours) and the 5 reactions were stopped by adding samples to the same volume of 1 M Tris-HCl buffer at pH 7.0, since Tris is a known inhibitor of glucoamylase [Clarke & Svensson, 1984]. The production of glucose was determined by the glucose oxidase method [Rabbo & Terkildsen, 1960].
10 Initial rates of glucose production were determined by fitting the experimental data to the equation $c = At/(1+Bt)$, where c is the product concentration, t is time, and A (the initial rate) and B are obtained from the nonlinear regression. At 55°C, only the time
15 points before 70 hours were used for the calculations, since the glucose production by that time had already declined for wild-type GA.

Glucose condensations reactions: Glucose condensation reactions were performed at 35°C and 55°C 20 with 30% (w/v) D-glucose as substrate in 0.05 M acetate buffer at pH 4.4 for 12 days with the addition of 0.02% sodium azide, used to inhibit microbial growth in the reaction mixtures. The enzyme concentration was 2.64 μ M for both wild-type and mutant GAs. Samples were 25 taken at various times and the reactions were stopped by adding samples to the same volume of 1 M Tris-HCl buffer at pH 7.0. High Performance Thin Layer Chromatography (HPTLC) and Imaging Densitometry were used to determine the production of isomaltose by a 30 method modified from that described by Robyt et al. [Robyt and Mukerjea, 1994]. One microliter of variously diluted samples and six different concentrations of standard (containing glucose, maltose and isomaltose) were applied to the HPTLC plates. The 35 developing solvent system contained acetonitrile, ethyl acetate, 1-propanol and water in the volume proportions

of 85:20:50:40. Only one ascent was used to develop the carbohydrate separation on HPTLC plates. After development, the plates were air-dried, dipped into an EtOH solution containing 0.3% (w/v) alpha-naphthol and 5 5% (v/v) H_2SO_4 , air-dried again, and incubated approximately 10 min at 120°C to visualize the carbohydrates. Densities of the isomaltose spots on HPTLC plates were quantified by Imaging Densitometry (Bio-Rad, Model GS-670), using Molecular Analyst 10 software (Bio-Rad). The experimental data were fitted to the equation $c = At/(1+Bt)$, described above for the hydrolysis of DE 10 maltodextrin, to obtain the initial rates of isomaltose production.

15

EXAMPLE 1**STABILIZATION OF *ASPERGILLUS AWAMORI* GLUCOAMYLASE
BY PROLINE SUBSTITUTION**

20 The following example is an exemplar of the methods and procedures that are used in the analysis of an individual mutation of a glucoamylase. To investigate the mechanisms governing *Aspergillus awamori* glucoamylase thermal stability, three proline 25 substitution mutations were constructed. These mutations were predicted to increase GA stability by decreasing the enzyme's conformational entropy of unfolding.

30 *Aspergillus awamori* glucoamylase (α -1,4-D-glucan glucohydrolase, EC 3.2.1.3; GA) is an enzyme which catalyses the release of β -glucose from the non-reducing ends of starch and related oligosaccharides. GA is used in, and defines the rate limiting step of, the commercial conversion of starch to high glucose 35 syrups which may be converted to fructose syrups by glucose isomerase, or used in fermentations to produce

ethanol. GA is used industrially at 55°-60°C; at higher temperatures the enzyme is rapidly and irreversible inactivated. Therefore, a GA variant with increased thermostability would be 5 advantageous industrially to decrease reaction times and/or to increase solids concentrations.

Previous work has shown that the natural stability of oligo 1,6-glucosidase [Suzuki et al., 1987] and pullulanase [Suzuki et al., 1991] can be positively 10 correlated to the mole percent proline present in the protein, and a general rule for protein stability has been proposed [Suzuki, 1989]. This work has been extended to show that bacteriophage T4 lysozyme [Matthews et al., 1987] and *Bacillus cereus* ATCC 7064 15 oligo 1,6 glucosidase [Watanabe et al., 1994] can be stabilized by engineering proline into selected sites thereby decreasing the protein's conformational entropy of unfolding.

Three sites (Ser30, Asp345 and Glu408→Pro) were 20 selected for proline substitution based on structural and evolutionary considerations. Mutations at these sites were constructed using the cloned *A. awamori* gene [Innis et al, 1985] and the proteins were expressed in *Saccharomyces cerevisiae* [Cole, et al, 1988]. The 25 stability of the mutant proteins was measured by their resistance to irreversible thermal inactivation at various temperatures. As shown herein, the Ser30→Pro mutation increased. However, unexpectedly the Glu408→Pro mutation decreased and the Asp345→Pro 30 mutation did not significantly alter GA stability.

Site-directed mutagenesis: Site-directed mutagenesis was performed as described herein above. The following oligonucleotides were used as mutagenic primers: CAGAGTCCGCGCCGGCACCCAAGCACCGTC (Ser30→Pro) 35 (SEQ ID No:3), AAGTCCAGCGACACAGGTGTGACCTCCAACGAC (Asp345→Pro) (SEQ ID No:4) and CGAGCGGAAAGCTGC

GGGCCATCAGACTTGTC (Glu408>Pro) (SEQ ID No:5).

Selection of sites for proline substitution:

Based on the nearly identical catalytic domain of *A. awamori* var *X100* GA whose structure is known [Aleshin et al, 1992] three sites for substitution were chosen, which met the following criteria: 1) Ramachandran (ϕ, ψ) angles were within allowed values for proline [Ramachandran et al., 1963]. For this work the ϕ and ψ angles at the substituted site were restrained to the broad range $\phi = -90^\circ$ to -40° , $\psi = 120^\circ$ to 180° or $\phi = -90^\circ$ to -40° , $\psi = -50^\circ$ to 10° . 2) Residues were highly solvent exposed, since mutation of residues in the core of the enzyme were thought to be more likely to decrease the enzyme's catalytic efficiency. 3) Residues didn't participate in hydrogen bonding with other amino acids. Additionally, based on sequence alignments with GA's from other organisms [Coutinho and Reilley, 1994b] only residues which met the above structural criteria and were not well conserved were selected for mutation. Ser30 could be aligned with proline in GAs from *Hormoconis grisea* var *thermoidea* and *H. resiae* GamP [Coutinho and Reilly, 1994b], which made it particularly attractive for proline substitution.

25

RESULTS

Specific activity

None of the proline substitution mutations significantly altered enzyme specific activities of wild-type and mutant GA's at 50°C and pH 4.5. This suggests that these mutations did not significantly alter the enzyme's structure around the active site or alter its interaction with substrate.

Irreversible thermal stability

35 Wild-type and mutant GA's were subjected to thermal inactivation at pH 4.5 as described in the

experimental protocols. Semilogarithmic plotting of the percent residual activity against inactivation time yielded inactivation rate coefficients (k_d). Figure 1 shows the relationship between temperature and k_d for 5 wild-type and mutant GA's. Based on these data, activation energies for thermal inactivation ($\Delta G'$) were calculated using transition state theory and melting temperatures (T_m), the temperature at which the enzyme was 50% inactivated after 10 minutes were computed 10 (Table 1). As can be seen, the Glu408 \rightarrow Pro mutation greatly decreased, the Asp345 \rightarrow Pro mutation did not significantly alter and the Ser30 \rightarrow Pro mutation increased GA stability.

It should be noted that although Table 1 shows 15 that the Asp345 \rightarrow Pro mutant GA demonstrated slightly increased $\Delta G'$ and T_m , these changes are generally not significant or that the Asp345 \rightarrow Pro mutant GA is more stable than wild-type since the k_{ds} for this mutant enzyme at two well separated temperatures (65° and 20 75°C) are essentially indistinguishable from wild-type (Figure 1).

The proline substitution mutations had different thermostabilities when measured by their resistance to irreversible thermal inactivation. When compared to 25 wild-type GA, Glu408 \rightarrow Pro decreased, Asp345 \rightarrow Pro did not significantly alter and Ser30 \rightarrow Pro increased GA stability (Figure 1 and Table 1).

Glu408 \rightarrow Pro destabilized GA. As was first suggested by Schimmel and Flory [1968] and has been 30 expanded by others [MacArthur and Thornton, 1991; Hurley et al, 1992] proline not only restricts the ϕ, ψ values for the site at which it exists, but also the ϕ, ψ values of the preceding residue. These reports suggest that the (ϕ, ψ) values for the residue preceding 35 proline should be restricted to approximately $\phi = -180^\circ$ to -55° and $\psi = 55^\circ$ to 180° or $\phi = -180^\circ$ to -55° and

ψ = -30° to -70° for all residues in Xaa-Pro except for Xaa-Gly, for which the preceding still applies, but is extended to include ϕ =45° to 180°. In the published *A. awamori* var. *X100* catalytic domain structure [Aleshin et al., 1992], Asp408 (ϕ =-65°, ψ =146°) which aligns with Glu408 in *A. awamori* GA, has ϕ, ψ values within ranges acceptable for proline. However, the preceding residue Gly407 (ϕ =80°, ψ =-5°) has ϕ, ψ outside acceptable ranges for positions preceding proline. It is not surprising then, that the Glu408 \rightarrow Pro destabilized GA. Additionally, X-ray crystallography suggests that position 408, in the closely related *A. awamori* var. *X100* GA², lies within a β -strand; a site not well suited for proline substitution.

Asp345 (ϕ = -65°, ψ = -26°) and the preceding Thr344 (ϕ = -116°, ψ = 178°) have ϕ, ψ angle values lay well within allowed values for proline substitution at position 345. However, the Asp345 \rightarrow Pro mutant GA did not demonstrate stability significantly different from wild-type GA. This is particularly unexpected since position 345 lies at the N-terminus of an α -helix²; a position previously shown to be particularly favorable for proline substitution [Watanabe et al, 1994].

Ser30 (ϕ = -49°, ψ = 130°) is preceded by Val29 (ϕ = -127°, ψ = 46°) both of which have acceptable ϕ, ψ angle values except Val29 ψ = 46° which is slightly smaller than ideal for proline substitution at position 30.

In summary, when expressed in *Saccharomyces cerevisiae*, Glu408 \rightarrow Pro greatly decreased, Asp345 \rightarrow Pro, did not significantly alter and Ser30 \rightarrow Pro strongly stabilized the enzyme. The Ser30 \rightarrow Pro mutant GA showed a significantly decreased rate of irreversible thermal inactivation when analyzed between 65° and 77.5°C without decreased enzyme activity. At 65°C a 1.7-fold decrease in thermal inactivation rate coefficients was

-30-

seen and the activation energy for thermal inactivation was increased by 1.6 kJ/mol relative to wild-type GA.

EXAMPLE 2

5 ENGINEERED DISULFIDE BONDS

The following example is an exemplar of the methods and procedures that are used in the analysis of an individual mutation of a glucoamylase. The process of GA thermoinactivation is thought to be dominated by 10 formation of enzymes with incorrect conformation [Munch and Tritsch, 1990]. Previous work supported this hypothesis. Site-directed mutagenesis has been used to eliminate sites of deamidation and peptide hydrolysis Chen et al., 1994 a,b). The corresponding mutations 15 Asn182→Ala and Asp257→Glu had reduced irreversible thermoinactivation rates at pH 4.5 below 70°C but increased rates above 70°C. Thus GA thermoinactivation is predominantly caused by "scrambled" structures rather than by deamidation and peptide hydrolysis. 20 Furthermore, mutations Gly137→Ala, Gly139→Ala and Gly137/139→Ala/Ala, made to reduce helix flexibility, showed increased thermostability up to 75°C (Chen et al., 1996) apparently by slowing down the formation of incorrect structures. 25 To improve protein thermostability by preventing formation of incorrect structures, several strategies have been proposed including introducing covalent linkage such as disulfide bonds (Perry and Wetzel, 1984; Wetzel, 1987; Matsumura et al., 1989, Clarke and 30 Fersht, 1993).

There are a total of nine cysteine residues in A. awamori GA, eight of which form disulfide-linked pairs, which are assumed to enhance the folding and stability of GA, residues 210 and 213, 262 and 270, 222 and 449 35 [Aleshin et al., 1992] and 509 and 604 [Williamson et al., 1992b]. In this Example, additional disulfide

bonds are introduced into GA to explore the effect on thermostability and catalytic activity. Two engineered disulfide bond mutants designated A27C/N20C (abbreviated S-S) and A471C/T72C were constructed. The new disulfide bond formed by A27C/N20C connects the C-terminus of helix 1 (Asn20) and a turn where residue Ala27 is located, while A471C/T72C bridges the N-terminus of helix 3 and the end of the 30-residue highly O-glycosylated belt region together. The disulfide bonds are formed spontaneously after fermentation and have different effects on GA thermostability and catalytic activity.

Site-Directed Mutagenesis: Site-directed mutagenesis was performed as described herein above.

15 Oligonucleotide primers used are: 5'-CGT ACT GCC ATC CTG TGT AAC ATC GGG GCG GA-3' (N20C, AAT→TGT) (SEQ ID No:6), 5'-ATC GGG GCG GAC GGT TGT TGG GTG TCG GGC GCG-3' (A27C, GCT→TGT) (SEQ ID No:7), 5'-CGA AAT GGA GAT TGC AGT CTC-3' (T72C, ACC→TGC) (SEQ ID No:8), 5'-G AGT ATC GTG TGT ACT GGC GGC ACC-3' (A471C, GCT→TGT) (SEQ ID No:9), with the underlined letters indicating the nucleotide mutations.

SDS-PAGE and Thio-titration: SDS-PAGE was carried out using 0.75 mm thick 10% polyacrylamide gels following the method of Garfin [1990]. For thio-titration, GA at 2 mg/ml concentration was denatured by boiling in denaturing solution containing 2% SDS, 0.08 M sodium phosphate (pH 8.0) and 0.5 mg/ml EDTA [Habeeb, 1972] with or without 50 mM DTT [Pollitt and Zalkin, 1983] for 10 min. The denatured GA (reduced or non-reduced) was concentrated using Centricon 30 concentrators (Amicon, MA, USA) and the reduced GA was applied to Bio-spin 30 chromatography columns (Bio-Rad, CA, USA) pre-equilibrated with denaturing solution to remove DTT. The resulting solution as well as the non-reduced denatured GA sample were divided into two portions.

One portion was used for a protein concentration assay and the other portion was assayed for thio reduction by mixing with 4 mg/ml DTNB in denaturing solution with a 30:1 volume ratio, followed by incubation at room 5 temperature for 15 minutes, and absorbance measurement at 412 nm with a molar absorptive value of $13,600 \text{ M}^{-1}\text{cm}^{-1}$ [Habeeb, 1972].

GA Activity Assay: As described herein above, maltose was used as substrate in enzyme kinetics 10 studies, with concentrations ranging from 0.2 K_m to 4 K_m at 35°C and pH 4.5 as described previously [Chen et al., 1994b]. Kinetics parameters were analyzed by the program ENZFITTER. In residual enzyme activity assays, the conditions are the same as in the enzyme kinetics 15 studies except that only one concentration of maltose (4%) is used as substrate. Specific activity assays were carried out with 4% maltose as substrate at 50°C and pH 4.5. One unit (IU) was defined as the amount of enzyme required to produce 1 μmol glucose per min under 20 the conditions of the assay. To compare the temperature optima of catalytic activities of wild-type and mutant GA, activities were assayed at pH 4.5 with 4% maltose as substrate at different temperatures.

Irreversible Thermoactivation: As described 25 herein above, purified wild-type or mutant GA proteins were incubated at five different temperatures from 65°C to 75°C at 2.5°C intervals at 40 $\mu\text{g}/\text{ml}$ in 0.05 M NaOAC buffer (pH 4.5). At six different time points, aliquots of the incubating enzyme were removed, quickly 30 chilled on ice, stored at 4°C for 24 hours, and subjected to residual activity assay. The irreversible thermoactivation of GA obeyed first-order kinetics [Chen et al., 1994b]. Thermoactivation rate coefficients, k_d , were determined as described previously 35 [Chen et al., 1994b].

Mutated Residues: The candidate residues of *A. awamori* GA to form disulfide bond were modeled with the crystal structure of *A. awamori* var. X100 GA [Aleshin et al., 1992] (1gly in the Brookhaven Protein Data Bank) as reference by the SSBOND program (Hazes and Dijkstra, 1988) installed in a DEC 3100 workstation.

Selection of Mutation Site: Residues Asn20, Ala27 and Thr72, Ala471 were chosen to be replaced with cysteine. After the analysis of crystal structure of *A. awamori* var. X100 GA [Aleshin et al., 1992] by the program SSBOND, 132 pairs of residues were found that could potentially be sites for a disulfide bond. Pairs containing glycine were discarded on the assumption that glycine may be required for flexibility at that site. Also, the residues involved in hydrogen bonds and electrostatic interactions were eliminated.

Residues 20 paired with 27 as well as 72 paired with 471 were chosen as candidates for disulfide bond formation according to the geometrical analysis. Amino acid sequence alignment among related GAs showed that there is a disulfide bond between position 20 and 27 in *Neurospora crassa* [Coutinho and Reilly, 1994b], which suggested that introducing disulfide bond between position 20 and 27 would not cause unfavored interactions there in *A. awamori* GA. Furthermore, the 20/27 disulfide bond would link the C-terminus of helix 1 and the conserved S1 fragment of GA involved in substrate binding [Coutinho and Reilly, 1994a] to form a loop, near another loop very critical for catalysis containing Trp 120, a residue involved in substrate binding [Sierks et al., 1989]. Therefore, the proposed 20/27 disulfide bond was expected to stabilize GA by keeping the correct conformation for catalysis and substrate binding.

Another further candidate for a disulfide bond pair was between positions 471 and 72. This disulfide

bond would link the N-terminus of helix 3 and the end of the 30-residue (440-470) highly O-glycosylated belt region to form a loop. This disulfide bond also would make an additional linkage between the catalytic domain 5 and the O-glycosylated linker. This O-glycosylated linker has been proved to be important for GA thermostability by limiting the conformational space available to the GA unfolded peptide [Semimaru et al., 1995 and Williamson et al., 1992]. This disulfide bond 10 could have a globe effect on the thermostability of GA because of this linkage. The side chain -OH group of Thr72 in *A. awamori* var. X100 GA [Aleshin et al., 1992] is hydrogen bonded to the main chain N atom of Asp73. In *A. awamori* GA, however serine is found at residue 73 15 in place of Asp. It is possible that the hydrogen bond between residues 72 and 73 does not exist in *A. awamori* GA, and therefore replacing Thr72 with Cys will not disturb this interaction. This hydrogen bond is apparently not critical for GA since Thr72 is replaced 20 by Ala, Lys or Val in other GAs [Coutinho and Reilly, 1994b].

Engineered Disulfide Bonds Were Formed

Spontaneously: After GA purification, the engineered disulfide bonds were found to be formed spontaneously 25 by the following two approaches.

First, the mutant A471C/T72C has faster mobility than wild-type during SDS-PAGE under non-reducing conditions, suggesting that an additional disulfide bond forms a new loop retarding the migration. The 30 possibility that a truncated enzyme was formed in this case was eliminated by DNA sequencing of the mutant cDNA and MALDI analysis. The MALDI data showed that the mutant GA had the same molecular weight as wild-type GA. Mutant A27C/N20C has the same migration as 35 wild-type GA, which may be because the additional loop caused by the engineered disulfide bond is too small.

(seven residues) to affect migration.

Second, the new disulfide bonds were demonstrated by thio group titration. Comparing the numbers of free thio groups before and after the treatment of reducing reagent DTT, the total disulfide bonds in mutant and wild-type GA were deduced as reported in Table 2. Wild-type, A27C/N20C, and A417C/T72C GA have in total 8.6, 10.9, and 10.4 free thio groups respectively according to the [SH]/molecule ratio in the presence of reducing reagent DTT (Table 2). In the absence of DTT the numbers are 0.9, 0.9 and 1.3, respectively (Table 2). This suggested that the number of disulfide bonds among wild-type, A27C/N20C and A471C/T72C are 4, 5 and 5, respectively. Therefore, the introduced cysteine residues formed disulfide bonds instead of remaining free thiols.

Enzymatic Activity and Optima Temperature of Catalysis: The enzymatic properties of double mutations A27C/N20C and A471C/T72C were not changed compared with wild-type at 35°C and 50°C as shown in Table 3, while single mutations had significant reduced activity. Mutant A27C/N20C and A471C/T72C had specific activities at 50°C and kinetic parameters at 35°C very close to wild-type GA (Table 3). The single mutant A27C had slightly increased K_m but the same k_{cat} value as wild-type GA, and thus a reduced k_{cat}/K_m ratio of ~30%. Mutant N20C had the same K_m but both a decreased k_{cat} and k_{cat}/K_m ratio and a decreased specific activity at 50°C of more than 50%.

Irreversible Thermoactivation of GA: The irreversible thermoactivation of wild-type and mutant GA was studied at 65°C, 67.5°C, 70°C, 72.5°C and 77.5°C with first-order irreversible theremoactivation coefficients k_d shown in Figure 2. Mutants A27C, A27C/N20C and A471C/T72C had smaller k_d values than did wild-type GA within the measured temperature range,

which means the activity decayed more slowly than wild type, while mutant N20C had greater k_d value than wild-type at all temperatures except 75°C, which means that N20C decayed faster than wild-type.

5 Table 4 shows the activation enthalpy (ΔH_f), entropy (ΔS_f) and free energy of unfolding (ΔG_f) at 65°C and 75°C of wild-type and mutant GAs, calculated according to transition-state theory. The enthalpies of N20C and A27C/N20C decreased by 42 and 24 KJ/mol respectively, while no significant change occurs for A27C and A471C/T72C. Mutants N20C and A27C/N20C had decreased entropy of 115 kJ/mol and 75 kJ/mol respectively, while entropy of mutants A27C and A471C/T72C showed no significant change. Mutant A27C
10 and A471C/T72C had a slightly higher $\Delta G'$ than wild-type GA at 65°C and 75°C (<0.5 kJ/mol), while the $\Delta G'$ of A27C/N20C was higher than that of wild-type by 1.5 and 2.2 kJ/mol at 65°C and 75°C respectively. Mutant N20C had a decreased $\Delta G'$ by 3.0 and 1.8 kJ/mol at 65°C and
15 75°C, respectively, compared with wild-type GA. Therefore, the engineered disulfide bond mutant A27C/N20C significantly increased GA thermostability compared with wild-type GA while the single mutants produced either a slight increase (A27C) or a slight
20 decrease (N20C) in thermostability. The other disulfide bond mutant had the thermostability identical to wild-type GA.

25

EXAMPLE 3

MUTATION A27C/N20C IN COMBINATION WITH OTHER MUTATIONS

30

In previous studies applicants have constructed the thermostable mutants G137A [Chen et al., 1996] and S436P (Li et al., 1996), which have the potential to be combined and improve thermostability additively. In
35 this Example, these mutations are combined with each other and with A27C/N20C (S-S; Example 2) to test their

effects (cumulative/additive) on thermostability and GA activity.

Enzymatic Activity and Optima Temperature of

Catalysis: The combined mutants A27C/N20C/G137A and 5 A27C/N20C/S436P had increased specific activity while mutant G137A/S436P had similar specific activity to wild-type GA (Table 3). The double mutants A27C/N20C and A471C/T72C as well as the combined mutant A27C/N20C/G137 had changed optimal temperatures for 10 catalysis.

Relative activity assays at temperatures from 60°C to 74°C (Figure 3) showed that wild-type, mutant A27C/N20C and A471C/T72C had the highest activity at 15 71°C, 72°C and 72.5°C, respectively. From 60°C to 67.5°C, mutant and wild-type GA had very similar activities. However, when the temperature was above 70°C, their relative activities differed substantially. Mutants A27C/N20C and A27C/N20C/G137A had higher 20 activity than wild-type consistently from 70°C to 76°C with a peak at 72.5°C, while mutant A471C/T72C had activity lower than wild-type from 70°C to 71°C and 73°C to 74°C but higher at 72°C which is its optimal 25 temperature. Thus mutant GAs A27C/N20C, A471C/T72C and the combined mutant A27C/N20C/G137A had increased temperature optima above wild-type GA by 1.5°C.

Irreversible Thermoactivation of GA: The irreversible thermoactivation of wild-type and mutant GA was studied at 65°C, 67.5°C, 70°C, 72.5°C and 77.5°C with first-order irreversible thermoactivation 30 coefficients k_d shown in Figure 2. Mutants A27C, A27C/N20C and A471C/T72C, A27C/N20C/G137A, A27C/N20C/S436P and G137A/S436P had smaller k_d values than did wild-type GA within the measured temperature range, which means the activity decayed more slowly 35 than wild type, while mutant N20C had greater k_d value than wild-type at all temperatures except 75°C, which

means that N20C decayed faster than wild-type.

Table 4 shows the activation enthalpy (ΔH_f), entropy (ΔS_f) and free energy of unfolding (ΔG_f) at 65°C and 75°C of wild-type and mutant GAs, calculated according to transition-state theory.

The helix flexibility mutant G137A showed additive thermostability when combined with either S436P or A27C/N20C. The combination S436P with A27C/N20C did not show additivity.

10

EXAMPLE 4

FURTHER STUDIES WITH COMBINED MUTATIONS

To further investigate whether individual stabilizing mutations can cumulatively stabilize *Aspergillus awamori* glucoamylase (GA), mutant enzymes were constructed containing combinations of thermostabilizing mutations. Previous work has shown that the following mutations stabilize GA as demonstrated by decreased irreversible thermal inactivation rates when inactivated in the absence of carbohydrate: Ser30→Pro (S30P; Example 1), Gly137→Ala (G137A), and Asn20→Cys/Ala27→Cys (which creates a disulfide bond between residues 20 and 27 and is therefore noted as S-S for convenience; Example 2). To investigate whether individual stabilizing mutations can cumulatively stabilize GA, additional combined mutant enzymes were prepared utilizing the these three mutations.

Site-directed mutagenesis: The S-S/S30P/G137A combined mutant was constructed using the S-S/S30P oligonucleotide listed above and a single stranded DNA template derived from a pBluescript II KS(+) vector with a 1.7 kb XhoI→BamHI DNA fragment coding for the GA catalytic domain which already contained mutations conferring the S30P and G137A amino acid substitutions. The presence of the individual mutations was confirmed

by sequencing and each mutated GA gene fragment was subcloned into YEPM18 [Cole et al., 1988] and transformed into *S. cerevisiae*.

5 **Thiol analysis:** 10 nmol of wild-type, S-S/S30P and S-S/S30P/G137A mutant GAs were incubated in 0.2 mM 5,5'-dithiobis(2-nitrobenzoic acid), 6 M GdnHCl, and 50mM Tris, pH 8 in duplicate [Fierobe et al., 1996]. The thiol concentration was calculated from a standard curve established using 0-30 μ M cysteine.

10 **Irreversible thermal inactivation:** Wild-type and mutant GAs were subjected to thermal inactivation at six or seven temperatures between 65° and 80°C at 2.5°C intervals in duplicate. Following 24 hours at 4°C, the residual activities of the inactivated samples were 15 analyzed at 35°C along with a corresponding sample which had not been inactivated [Chen et al, 1996].

15 **Saccharification analysis:** Saccharifications were performed in duplicate using stirring heating blocks and tightly sealed vials to prevent evaporation. Eight 20 μ g/ml of wild-type and mutant GAs were assayed using 28% (w/v) Maltrin DE 10 maltodextrin in 0.05 M NaOAc pH 4.5 as substrate. At various times, sample was removed, diluted appropriately in 0.05 M NaOAc pH 4.5 and the reaction stopped by adding 100 μ l of diluted 25 sample to 40 μ l of 4.0 M Tris-Cl, pH 7.0. The glucose concentration was determined by a glucose oxidase/o dianisidine assay [Banks and Greenwood, 1971].

RESULTS

Enzyme activities

30 Table 5 shows the specific activities of the wild-type and mutant GAs at 50°C and pH 4.5 using maltose as substrate. None of the mutant GAs demonstrated reduced enzyme activity and the S30P/G137A and S-S/S30P/G137A mutants were somewhat more active than wild-type at 35 50°C. To further investigate this observation, the activities of these mutant enzymes were assayed at

various temperatures between 35° and 68°C (Figure 4). The S30P/G137A and S-S/S30P/G137A mutant GAs were more active than wild-type at all temperatures examined.

Thiol analysis

5 The formation of a disulfide bond between positions 20 and 27 in the Asn20→Cys/Ala27→Cys mutant GA has been confirmed (Example 2). Table 6 shows the results of thiol analysis for the combined mutants S-S30P and S-S/S30P/G137A. *A. awamori* GA has one free 10 cysteine at position 320. The combined mutant GAs show slightly higher thiol content per molecule than wild-type which may reflect less than complete disulfide bond formation between positions 20 and 27. However, if the disulfide bond was completely unformed, the 15 [SH]/protein would be expected to rise to approximately three with the addition of two free cysteine residues. Therefore, we conclude that the disulfide bridge is formed at 70-80% the expected theoretical yield for complete formation.

20 *Irreversible thermal inactivation*

Wild-type and mutant GAs were subjected to thermal inactivation at pH 4.5 between 65° and 80°C. Semilogarithmic plotting of residual activity versus inactivation time yielded inactivation rate 25 coefficients (k_d). Figure 5 shows the effect of temperature on k_d for wild-type and mutant GAs. As can be seen, the combined mutants are significantly more stable than the individual mutant enzymes. Additionally, the temperature at which the enzymes were 30 50% inactivated after 10 minutes (T_m) was calculated by extrapolation from the thermal inactivation plots and transition state theory was used to calculate activation energies for thermal inactivation ($\Delta G'$). Table 7 shows the changes in $\Delta G'$ ($\Delta\Delta G'$) and T_m for the 35 combined mutant GAs relative to wild-type GA. These data clearly demonstrate that combining the individual

stabilizing mutations can cumulatively stabilize the enzyme.

Saccharification analysis

Figure 6 shows the results of saccharification analysis at 55° and 65°C for wild-type, S30P/G137A and S-S/S30P/G137A GAs using the industrial DE 10 maltodextrin substrate Maltrin M100 (28% w/v) from Grain Processing Corporation. Complete conversion of 28% w/v DE 10 maltodextrin to glucose would result in a 1.71 M glucose syrup however, previous saccharification analyses in our laboratory have demonstrated that wild-type GA results in approximately 90% theoretical maximum glucose yield at 55°C (not shown). At 55°C no significant difference in glucose production was observed between the wild-type and mutant enzymes. However, at 65°C the mutant GAs produced 8-10% more glucose than wild-type although none of the enzymes tested produced as much glucose as at 55°C probably due to thermal inactivation at the elevated reaction temperature.

In summary these data show that the S30P/G137A double mutant enzyme was more stable than either single mutant GA when analyzed for resistance to irreversible thermal inactivation between 65°C and 80°C. The S-S/S30P combined GA mutant was also more stable than either the S30P or the S-S mutant GAs. The S-S/S30P/G137A combined mutant was the most stable GA variant constructed, particularly at temperatures above 70°C when inactivated in a buffer system lacking mono- or polysaccharides. Saccharification analysis showed that the mutant enzymes performed better at elevated temperatures than wild-type GA. Importantly, none of the combined mutant GAs showed decreased enzyme activity when analyzed at 50°C.

35 Discussion

Sites of mutation

As described in Example 2, the mutations Asn20→Cys and Ala27→Cys form a disulfide bond between the C-terminus of α -helix one and an extended loop between α -helices one and two. S30P and G137A were designed to 5 stabilize the enzyme by reducing its conformational entropy of unfolding and are the most stabilizing in a series of proline substitution (Xaa→Pro) and Gly→Ala mutations respectively. Ser30 is located at the second 10 position of a type II β -turn on an extended loop between α -helices one and two and Gly137 is located in the middle of the fourth α -helix.

It is of particular importance to note the positions of the S30P and the disulfide bond forming mutations. The disulfide bond is formed between 15 positions 20 and 27; relatively close to position 30. The fact that both the disulfide bond forming mutations and the S30P stabilize GA suggests that this region of the enzyme is critical for irreversible thermal inactivation and may represent a region of local 20 unfolding important for thermal inactivation.

Additionally, previous investigators have suggested that a disulfide bond should not be engineered within four amino acids of a proline in primary sequence [Balaji et al, 1989]. This Example demonstrates that 25 this rule is not absolute since thiol analysis showed that the disulfide bond was formed in the S-S/S30P and S-S/S30P/G137A combined mutants and thermal inactivation studies showed the stabilizing effects of the mutations were cumulative.

30 **Cumulative stabilization**

Previous work by Applicants has shown that combining two stabilizing mutations does not necessarily stabilize GA [Chen et al, 1996]. The present study, however, demonstrates that combining 35 stabilizing mutations, even mutations very close to each other in the protein, can cumulatively stabilize

GA as measured by resistance to irreversible thermal inactivation.

The S30P/G137A mutant showed more than additive stabilization at low temperatures (65°-70°C), but less than additive stabilization at high temperatures (77.5°-80°C) (Figure 5A and Table 7). At 80°C the inactivation rate for the S30P/G137A combined mutant was nearly identical to the S30P individual mutant protein. This indicates that both regions are very important for low temperature thermal inactivation, but at high temperatures inactivation became governed by other processes.

It was somewhat surprising that combining the S30P with the disulfide bond forming mutations resulted in cumulative stabilization. This is not only because the engineered disulfide bond is so close to the engineered proline as is discussed above, but also because both are targeting the same region of the protein (ie: the extended loop between α -helices one and two). It was expected that either the disulfide bond or S30P stabilized this region maximally, and further stabilization at this site would not result in a functionally more stable enzyme. As can be seen in Figure 5B, this was not the case. Combining the mutations resulted in roughly additive stabilization at all temperatures examined between 65° and 80°C.

The S-S/S30P/G137A combined mutant was no more stable than S30P/G137A GA at low temperatures (65°-70°C), but was slightly more stable at higher temperatures (75°-80°C) (Figure 5C and Table 7). Interestingly, the S-S/S30P GA is also more stable than S30P/G137A GA at high temperatures. Therefore, it appears that the introduced disulfide bond is particularly effective at stabilizing GA at high temperatures.

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EXAMPLE 5
INDUSTRIAL APPLICATION

To determine whether the thermal stabilizing mutations: S30P/G137A and S-S/S30P/G137A would enhance 5 GA performance under industrial conditions wild-type and mutant enzymes were subjected to high temperature saccharifications (Figure 6). Saccharification analysis showed that the mutant enzymes out-performed wild-type at 65°C but not at 55°C likely due to their 10 increased stability.

Conclusion

The S30P/G137A double mutant cumulatively stabilized GA as demonstrated by decreased irreversible thermal inactivation rates relative to either 15 individual mutant enzyme when analyzed between 65°C and 80°C. Similarly, the S-S/S30P combined mutant also demonstrated cumulative stabilization. The S-S/S30P/G137A combined mutant was more stable than either of the "double" mutants, particularly at 20 temperatures above 70°C. The S-S/S30P combined mutant had the same activity as wild-type and the S30P/G137A and S-S/S30P/G137A mutants increased enzyme activity by 10-20% when assayed between 35° and 68°C. The S30P/G137A and S-S/S30P/G137A mutant GAs decreased 25 thermal inactivation rates approximately three fold relative to wild-type when inactivated in the presence of 1.71M glucose at 65°C. Additionally, at 55°C no difference in glucose yield was observed between these mutant GAs and wild-type for the saccharification of 30 the industrial substrate Maltrin M100, whereas at 65°C the S30P/G137A and S-S/S30P/G137A GAs produced 8-10% more glucose than wild-type.

EXAMPLE 6

35 **MUTATIONS WITH INCREASED SELECTIVITY**

Interactions between substrates and charged

residues at subsites 1 and 2 of GA play a very important role in substrate specificity since the catalytic site is located between these sites. Therefore mutations were designed and analyzed to 5 determine residues within these regions where mutations would increase selectivity of the enzyme reaction. In addition, several mutations that were designed to have thermostability were also screened for selectivity, as well as mutations designed to increase the pH optimum.

10 *Site-directed mutagenesis:* Site-directed mutagenesis was performed as described herein above. The following mutagenic oligonucleotide primers were synthesized at the Iowa State University Nucleic Acid Facility: 5'-GGT CTC GGT GAG CCC AGG TTC AAT GTC GAT-3' (Lys108→Arg; SEQ ID No:10), 5'-GGT CTC GGT GAG CCC ATG TTC AAT GTC GAT-3' (Lys108→Met; SEQ ID No:11), 5'-GAG GAC ACG TAC TGG AAC GGC AAC CCG-3' (Tyr312→Trp; SEQ ID No:12), and 5'-TAC CCT GAG GAC ACG TAC AAC GGC AAC GGC AAC TCG CAG GGC AAC CCG TGG TTC CTG TGC-3' (311-314 Loop; SEQ ID No:13), the underlined letters indicating the changed or added nucleotides.

Results

Enzyme kinetics

As shown in table 11, the kinetic parameters k_{cat} 25 and K_m for the hydrolysis of G_2 to G , as well as iG_2 in 0.05 M acetate buffer, pH 4.4, at 45°C are given in Table 8. The 311-314 Loop mutant had k_{cat} values 50-80% for all α -(1,4)-linked substrates and only 30% for iG_2 , K_m values 50-75% for all substrates. The k_{cat} values for 30 Gly137→Ala/Ser30→Pro GA are 10-30% more, generally, than that of wild-type GA for all substrates. The K_m values of Gly137→Ala/Ser30→Pro GA are about half to twofold for all the α -(1,4)-linked substrates and essentially reached the wildtype level for iG_2 . The k_{cat} 35 values for the GA engineered to carry the triple mutation, S-S/Gly137→Ala/Ser30→Pro, ranged from 80 to

120% generally for all substrates, and the K_m values are 30-80% for all substrates compared to wild-type GA. The k_{cat} values for S-S GA are 85-110% for all substrates, and the S-S GA K_m values are generally 90-110% for all substrates. However, the S-S GA K_m values are 140% for G₅ and 190% for G₆. Values of k_{cat}/K_m are 75-105%, 60-110%, 60-110%, and 60-120% for the 5 Tyr312→Trp mutation, the combined Ser30→Pro/Gly137→Ala double mutation, the combined S-S/Ser30→Pro/Gly137→Ala 10 triple mutation, and the S-S engineered GA, respectively. The catalytic efficiencies for the 311-314 Loop GA are 85-120% for all the α -(1,4)-linked substrates, and only 50% for iG₂, compared to wild-type GA.

15 Table 8 shows the ratios of the catalytic efficiencies for G₂ to iG₂ for wild-type and mutant GAs. GAs engineered with the 311-314Loop mutation and Lys108→Arg mutation have the highest (240%) and the lowest (20%) catalytic efficiencies for α -(1,4)- over 20 α -(1,6)-linked substrates, respectively. The GAs engineered with the Tyr312→Trp and S-S mutations show 50% and 20% increases for this ratio, respectively. All other mutants had lower ratios, indicating poorer α -(1,4)-hydrolytic ability relative to α -(1,6)-hydrolytic ability than wild-type GA.

Maltooligosaccharide hydrolysis

GA engineered with the 311-314Loop mutation or with the S-S mutation had the highest average glucose yields (Figure 7). The 311-314Loop GA had the lowest 30 initial rates for glucose production (64%, 61%, and 82% compared to wild-type GA at 35, 45, and 55°C, respectively) due to a specific activity only 60% that of wild-type GA (data not shown). Glucose concentrations decreased after reaching maximal values 35 because of conversion to oligosaccharides.

Glucose condensation reactions

IG₂ concentration profiles in 30% (w/v) glucose condensation reactions at 35, 45, and 55°C were analyzed. GAs engineered with the Lys108Arg mutation had the highest and the 311-314Loop mutation as well as 5 the S-S mutation the lowest equilibrium iG₂ concentrations at all three temperatures. Tyr312→Trp, Ser30→Pro/Gly137→Ala, and S-S/Ser30→Pro/Gly137→Ala GAs exhibited essentially the same equilibrium iG₂ concentrations as wild-type GA. For all the other 10 engineered thermostable GAs tested, Ser436→Pro, S-S/Ser436→Pro, S-S/Gly137→Ala, and Gly137→Ala/Ser436→Pro, all reached higher equilibrium iG₂ concentrations than did wild-type GA. Table 9 shows 15 the initial rates of iG₂ formation in 30% (w/v) glucose condensation reactions. S-S and 311-314Loop mutant GAs have the lowest initial rates at all three reaction temperatures tested. Lys108→Arg mutant GA showed the highest initial rates among all the mutant GAs tested at all three reaction temperatures. All the tested 20 thermostable GAs except Ser30→Pro/Gly137→Ala and S-S/Ser30→Pro/Gly137→Ala had much higher initial rates than wild-type GA at 35°C, but they dropped to slightly higher or almost the same rate as wild-type GA at 55°C. The specificity for α -(1,6)-linkage synthesis over α - 25 (1,4)-linkage hydrolysis

The ratio of the initial rate of iG₂ production in a 30% (w/v) glucose condensation reaction to that of glucose formation in 30% DE 10 maltodextrin hydrolysis was calculated to estimate the selectivity for the 30 synthesis of α -(1,6)-linked products over the hydrolysis of α -(1,6)-linked substrates. These iG₂/glucose ratios and their relative ratios for wild-type and mutant GAs are given in Table 9. K108R and S-S mutants showed the highest and the lowest relative 35 ratios among wildtype and all the mutant GAs at all reaction temperatures, respectively. Therefore, K108R

had more specificity for α -(1,6)-linkages than α -(1,4)-linkages and S-S GA had more affinity for α -(1,4)-linkages than α -(1,6)-linkages. The 311-314Loop GA also showed very low relative ratios at these three

5 temperatures.

EXAMPLE 7

ADDITIONAL SELECTIVITY MUTATION ANALYSIS

Utilizing the methods as set forth herein above,
10 additional mutations were screened for selectivity as shown in Table 10 and Figures 8 and 9.

Enzyme Kinetics: The kinetic parameters are seen in (k_{cat} and K_m) for the hydrolysis of α -1,6-linked isomaltose and α -1,4-linked maltooligosaccharides (DP2-7)
15 at 45°C and pH 4.4 are given in Table 10. Mutant Y175F was active. The k_{cat} and K_m values were 83-141% and 106-171%, respectively, that of wildtype for the different substrates tested and catalytic efficiencies were 69-102% that of wildtype. Mutant R241K was also active.
20 Mutant S411G was highly active. The k_{cat} and K_m values were 93-129% and 83-203%, respectively, that of wildtype for the different substrates tested and catalytic efficiencies were 55-122% that of wildtype. Mutant S411A had a similar catalytic efficiency ratio
25 as wildtype. Mutants Y116W, R241K, and S411G had decreased catalytic efficiency ratios compared to that of wildtype GA.

DE 10 Maltodextrin Hydrolysis: At 55°C, the highest glucose yield was about 95% reached by engineered GA
30 with mutant S411A at 216 hours compared to the wildtype yield of about 90% (Fig. 9). All of the GAs, except S411A, reached their highest glucose yields rapidly. The glucose yield of S411A slowly increased for an extended period of time. The initial rates of glucose
35 production at 55°C were 5 to 8 times higher than those at 35°C.

5 **Glucose Condensation Reaction:** Glucose condensation reactions were used to study the ability of wildtype and mutant GAs to synthesize isomaltose at high glucose concentrations (Figure 8). The same concentrations of glucoamylases (2.64 μ M) were used as in the hydrolysis of DE 10 Maltodextrin.

At 55°C, in spite of the different initial rates of isomaltose production for wildtype, R241K and Y175F, isomaltose production reached almost the same 10 concentration at the last time point for these three mutant GAs (Figure 8), indicating that the isomaltose production was close to equilibrium status. Isomaltose production for S411A and S411G was much lower than wildtype and almost linear as it was also at 35°C. 15 Unexpectedly, isomaltose production for Y116W had a different (lower) equilibrium status compared to wildtype. The initial rates of isomaltose production at 55°C were 5 to 7 times greater than those at 35°C. R241K had a decreased initial rate of isomaltose 20 production at 55°C compared to that of wildtype, and it also had a lower increase (about 5 times) in the initial rate of isomaltose production from 35°C to 55°C, compared to the wildtype increase (about 7 times). Y116W, Y175F, S411A and S411G had increased 25 initial rates of isomaltose production of about 7, 6, and 5 times, respectively from 35°C to 55°C.

30 **Selectivity:** The ratio of the initial rate of isomaltose production (from glucose condensation reactions) to that of glucose production (from hydrolysis of DE 10 maltodextrin) was calculated to evaluate selectivity for the synthesis of α -1,6-linked products versus the hydrolysis of α -1,4 linked substrates. This ratio represents the ability of a GA to synthesize isomaltose at a normalized level of DE 10 35 maltodextrin hydrolytic activity.

Mutants Y175F, S411A and S411G had a decreased

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ratio of the initial rate of isomaltose production to that of glucose production to that of glucose production by 12%, 35% and 56% at 35°C, respectively, and a decreased ratio by 24%, 60% and 62% at 55°C,
5 respectively, compared to wildtype. R241K had a very similar ratio to that of wildtype at both 35°C and 55°C.

EXAMPLE 8

10 MUTATIONS TO PROVIDE pH OPTIMIZATION

Utilizing the methods as set forth herein above, additional mutations, S411G, S411A, S411C, S411H, S411D were screened for increased pH optimum as shown in Figure 10 and Tables 11 and 12.

15 Enzyme kinetics

The kinetic parameters, k_{cat} and K_m , for the hydrolysis of α -1,4-linked maltose and maltoheptaose and α -1,6-linked isomaltose at 45°C and pH 4.4 are given in Table 11. Mutant S411G glucoamylase was highly active compared to wild-type, with an increased k_{cat} and K_m 13 - 30% and 11 - 59%, respectively, on the substrates tested. The catalytic efficiencies (k_{cat}/K_m) were 71 - 116% that of wild-type. Mutant S411A maintained 65 - 74% of wild-type catalytic efficiency with a slightly decreased k_{cat} and a slightly increased K_m . Mutant S411C maintained 54 - 73% of wild-type catalytic efficiency with a decrease in both the k_{cat} and K_m values. Since mutant S411H and S411D had only about 6 - 12% of wild-type catalytic efficiency resulting from a seriously decreased k_{cat} and an increased K_m , the kinetic parameters for the hydrolysis of isomaltose were not determined. Only mutant S411H and S411D had large increases (5.5 to 7.5 kJ/mol) in the transition-state energy, $\Delta(\Delta G)$, for the hydrolysis 35 of maltose and maltoheptaose. The large increases of transition-state energy indicated that the introduction

of histidine or aspartic acid into position 411 substantially destabilized the binding between GA and substrate in the transition-state.

pH dependence of GA activity

5 The kinetic parameters, k_{cat}/K_m and k_{cat} , of the hydrolysis of maltose by wild-type and mutant glucoamylases at different pH values were calculated from initial rates obtained at low (smaller than 0.2 K_m) and high (higher than 10 K_m) concentrations of maltose.

10 The effects of pH on the k_{cat}/K_m and k_{cat} of maltose hydrolysis were used to determine the pK values (Table 12) of both the free enzymes and the enzyme-substrate complexes. Although wild-type GA had a higher catalytic efficiency (k_{cat}/K_m) than all of the mutant

15 15 glucoamylases at all of the pH values tested, mutants S411G and S411A had higher k_{cat} values than that of wild-type at some pH values. The uncomplexed and maltose-complexed S411H and S411D showed more narrow bell-shaped curves than that of wild-type.

20 The effects of pH on the hydrolysis of maltoheptaose by wild-type, S411G and S411A GAs were measured to further investigate the change of pK values and optimum pH of enzyme-substrate complexes using a long-length substrate. Surprisingly, not only S411G,

25 but also S411A were highly active compared to wild-type at the optimum pH. Wild-type GA pK1 values (ionization of the catalytic base) were 2.77, 2.11, and 2.6 for the free enzyme, the maltose-complexed form, and the maltoheptaose-complexed form, respectively. The pK₂

30 values (ionization of the catalytic acid) of wild-type were 5.80, 5.85, and 6.78 for the free enzyme, the maltose-complexed form, and the maltoheptaose-complexed form, respectively [Bakir et al., 1993, Hiromi et al., 1966, Sierks and Svensson, 1994]. Compared to wild-

35 type, the S411G mutation increased the pK1 of both the maltose-complexed form and the maltoheptaose-complexed

form by approximately 0.6 units, whereas S411G had no effect on the pK₂ of either enzyme-substrate complexes and only had a minor effect on the pK₁ and pK₂ of the free enzyme. The combined effect of S411G on pK₁ and pK₂ was an increased optimum pH of both the maltose-complexed form and the maltoheptaose-complexed form by approximately 0.3 units.

The S411G mutation, however, had no effect on the optimum pH of the free enzyme. S411A and S411C had very similar effects on the pH dependence of maltose hydrolysis. S411A and S411C increased the pK₁ of the free enzyme and the maltose-complexed forms by 0.3 - 0.5 and 1.21 units, respectively. Surprisingly, S411A and S411C also increased the pK₂ of the maltose-complexed form by approximately 0.5 units. In addition, S411A increased the pK₁ and pK₂ of the maltoheptaose-complexed form by 1.31 and 0.4 units, respectively. S411H increased the pK₁ of the free enzyme and maltose-complexed form by 0.33 and 1.47 units, respectively; however, it decreased the pK₂ of the free enzyme and the maltose-complexed form by 0.79 and 1.16 units, respectively. S411D increased the pK₁ of the free enzyme and the maltose-complexed form by 0.36 and 1.23 units, respectively. S411D also decreased the pK₂ of the maltose-complexed form by 0.32 units. For wild-type, S411G, and S411A GAs, the values of pK₁, pK₂, and pH_{opt} for the maltoheptaose-complexed forms were higher than those of the corresponding maltose-complexed forms by approximately 0.5, 0.9 and 0.7 units, respectively. For S411G and S411A, the increases in pH optimum (compared to that of wild-type) obtained using the long-length substrate (maltoheptaose) were almost the same as that obtained using the short-length substrate (maltose).

All five mutants at position 411 showed a shift of 0.15 to 0.87 units in the optimum pH of the enzyme-

substrate complex compared to wild-type (Table 12), mainly due to increased pK_1 values. Compared with other mutants, S411A was the best performing pH mutant.

5 S411A increased the optimum pH by 0.84 units while also maintaining a high level of both catalytic activity (k_{cat}) and catalytic efficiency (k_{cat}/K_m).

The hydrolysis of maltodextrin 10

The hydrolysis of 28% (w/v) maltodextrin was used to study the pH dependence of GA activity at a high 10 concentration of a long-length substrate. Maltodextrin 10 is a mixture of maltodextrin with an average (and major) degree of polymerization of 10. The production of glucose by wild-type and S411A glucoamylases during the hydrolysis of maltodextrin 10 at 11 different pH 15 values was determined, and used to calculate the initial rates of glucose production at different pH values (Figure 10). The production of glucose increased following a hyperbolic curve. S411A had higher initial rates of glucose production than wild-type when the pH values were above 6.6 (Figure 10).

Throughout this application, various publications are referenced by author and year and 25 patents listed by number. Full citations for the publications are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

30 The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

35 Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood

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that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

TABLE 1

Changes in ΔG^\ddagger and Tm for the mutant GAs relative to wild-type.

GA form	$\Delta\Delta G^\ddagger$ (kJ/mol.)	ΔT_m (°C)
Ser30→Pro	1.6	1.7
Asp345→Pro	0.5	0.4
Glu408→Pro	-7.2	-6.7

TABLE 2

Summary of DTNB-titratable sulphydryl groups in wild-type and mutant GA with or without DTT reducing

Enzymes	[SH]/molecule		No. of disulfide bonds*
	DTT ⁺	DTT ⁻	
WT	8.6	0.9	4
A27C/N20C	10.9	0.9	5
A471C/T72C	10.4	1.3	5

* No. of disulfide bonds = ([SH]/molecule (DTT⁺)-[SH]/molecule (DTT⁻))/2

TABLE 3

Catalytic properties of wild type and mutant GAs.				
GA form	Specific Activity (IU/mg GA)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)
WT ^a	20.6±0.2 ^b	0.72±0.03	8.67±0.17	12.0
A27C	14.9±1.1	0.86±0.11	8.02±0.45	9.3
N20C	8.1±0.5	0.70±0.05	3.97±0.12	5.7
A27C/N20C	18.3±0.7	0.90±0.08	9.61±0.40	10.7
A471C/T72C	22.7±1.5	0.87±0.07	10.17±0.40	11.6
A27C/N20C /S436	22.5±1.8	N/D ^c	N/D	N/D
A27C/N20C /G137A	24.2±0.8	N/D	N/D	N/D
G137A/S436P	25.0±0.9	N/D	N/D	N/D

^aProduced in shaking flasks^bstandard error^cnot determined

TABLE 4

Activation parameters for irreversible thermoinactivation of wild-type (WT) and mutant GAs at pH 4.5.

GA form	ΔH^\ddagger (kJ/mol)	ΔS^\ddagger (J/mol·K)	$\Delta G^\ddagger (65^\circ\text{C})$ (kJ/mol)	$\Delta G^\ddagger (75^\circ\text{C})$ (kJ/mol)
WT ^a	366± 1 ^b	769± 4	105.7	98.0
A27C	370±15	780±44	106.3	98.5
N20C	324±11	654±33	102.7	96.2
A27C/N20C	342±16	694±46	107.2	100.2
A471C/T72C	365± 9	768±26	106.0	98.3
A27C/N20C /S436P	352± 6	724±18	107.9	100.7
A27C/N20C /G137A	362± 1	751± 2	108.4	100.9
G137A/S436P	362±20	752±57	107.7	100.2
S436P ^c	351± 8	723±24	106.2	99.0
G137A ^d	330± 6	661±17	106.5	99.9

^a produced by shaking flask

^b standard error

^c Li *et al.*, 1996

^d Chen *et al.*, 1996

Table 5. Specific activities of wild-type and mutant GAs

GA form	Specific activity ^a (IU/mg)
Wild-type	21.1±0.1
S30P/Gly137A	24.0±1.2
S-S/S30P	21.2±0.5
S-S/S30P/G137A	24.5±0.2

^a Standard deviation resulting from three or more assays

Table 6. Thiol analysis of wild-type and mutant GAs

GA form	[Protein] (μM)	[SH] (μM) ^a	[SH]/[Protein] ^a
Wild-type	10	8	0.8
S-S/S30P	10	11	1.1
S-S/S30P/G137A	10	13	1.3

^a Average of duplicate analysis

Table 7. Changes in free energies for thermal inactivation ($\Delta\Delta G^\ddagger$) and temperatures at which the enzyme is 50% inactivated after 10 minutes (ΔT_m) relative to wild-type GA

GA form	$\Delta\Delta G^\ddagger$ ^a (kJ/mol)	ΔT_m (°C)
S30P ^b	1.6	1.7
G137A ^c	0.8	1.2
S-S ^d	1.2	1.4
S30P/G137A	4.5	3.5
S-S/S30P	3.5	3.2
S-S/S30P/G137A	4.4	3.9

^a Calculated at 65°C

^b From Allen *et. al.*⁸

^c From Chen *et. al.*⁶

^d From Li *et. al.*⁷

Kinetic parameters of wild-type and mutant GAs for hydrolysis of maltoligosaccharides DP 2-7 (G2-G7) at 45°C in 0.05 M acetate

pH 4.4

Glucoamylase G₂ G₃ G₄ G₅ G₆ G₇**Wild-type**

k_{cat} (s ⁻¹)	18.6 ± 0.4 ^a	50.8 ± 0.6	67.5 ± 1.9	61.5 ± 0.33	65.9 ± 1.2	81.5 ± 1.8
K_M (mM)	1.09 ± 0.08	0.353 ± 0.013	0.239 ± 0.017	0.094 ± 0.002	0.098 ± 0.007	0.136 ± 0.009
k_{cat}/K_M (s ⁻¹ mM ⁻¹)	17.1 ± 0.9	144 ± 4	282 ± 13	653 ± 10	671 ± 36	599 ± 27

Lys108Arg

k_{cat} (s ⁻¹)	17.3 ± 0.5	32.6 ± 0.9	46.6 ± 1.6	51.7 ± 1.4	55.2 ± 1.4	86.2 ± 3.1
K_M (mM)	1.52 ± 0.11	0.570 ± 0.038	0.383 ± 0.029	0.307 ± 0.019	0.276 ± 0.016	0.481 ± 0.031
k_{cat}/K_M (s ⁻¹ mM ⁻¹)	11.4 ± 0.6	57.2 ± 2.5	122 ± 5	168 ± 6	200 ± 8	179 ± 6
$\Delta(\Delta G)^b$ (kJ mol ⁻¹)	0.92	2.10	1.91	3.08	2.75	2.74

Trp312Trp

k_{cat} (s ⁻¹)	17.2 ± 0.3	36.8 ± 0.9	50.7 ± 0.9	50.7 ± 0.8	56.0 ± 0.8	63.3 ± 0.6
K_M (mM)	0.940 ± 0.059	0.343 ± 0.028	0.193 ± 0.010	0.100 ± 0.006	0.108 ± 0.005	0.103 ± 0.003
k_{cat}/K_M (s ⁻¹ mM ⁻¹)	18.3 ± 0.90	107 ± 6	262 ± 9	508 ± 22	519 ± 20	617 ± 1
$\Delta(\Delta G)$ (kJ mol ⁻¹)	-0.16	0.67	0.17	0.57	0.58	-0.07

300Loop

k_{cat} (s ⁻¹)	14.7 ± 0.3	25.9 ± 0.6	34.1 ± 0.8	43.0 ± 0.6	41.4 ± 0.8	41.9 ± 0.7
K_M (mM)	0.738 ± 0.055	0.234 ± 0.019	0.114 ± 0.008	0.072 ± 0.004	0.064 ± 0.005	0.083 ± 0.005
k_{cat}/K_M (s ⁻¹ mM ⁻¹)	20.0 ± 1.2	111 ± 7	300 ± 17	598 ± 28	642 ± 47	506 ± 25
$\Delta(\Delta G)$ (kJ mol ⁻¹)	-0.35	0.60	-0.14	0.20	0.10	0.38

Ser30Pro/Gly137Ala

k_{cat} (s ⁻¹)	25.0 ± 1.1	50.2 ± 3.0	77.9 ± 2.2	77.7 ± 1.6	77.0 ± 2.2	80.3 ± 2.2
K_M (mM)	1.62 ± 0.11	0.596 ± 0.010	0.261 ± 0.020	0.175 ± 0.011	0.204 ± 0.017	0.151 ± 0.013
k_{cat}/K_M (s ⁻¹ mM ⁻¹)	15.5 ± 1.2	84.2 ± 3.1	299 ± 16	444 ± 21	377 ± 23	533 ± 37
$\Delta(\Delta G)$ (kJ mol ⁻¹)	0.27	1.42	-0.15	1.02	1.52	0.31

SS/Ser30Pro/Gly137Ala					
<i>k</i> _{cat} (s ⁻¹)	23.0 ± 0.9	42.1 ± 1.0	72.0 ± 2.1	72.2 ± 1.0	79.5 ± 1.7
<i>K</i> _M (mM)	1.66 ± 0.07	0.470 ± 0.032	0.236 ± 0.019	0.172 ± 0.007	0.157 ± 0.011
<i>k</i> _{cat} / <i>K</i> _M (s ⁻¹ mM ⁻¹)	13.9 ± 0.9	89.6 ± 4.2	305 ± 17	420 ± 13	505 ± 26
Δ(ΔG) (kJ mol ⁻¹)	0.55	1.26	-0.21	1.16	0.75
					1.00
 SS					
<i>k</i> _{cat} (s ⁻¹)	20.7 ± 0.6	40.8 ± 0.9	72.1 ± 1.3	76.5 ± 0.8	76.4 ± 2.1
<i>K</i> _M (mM)	1.16 ± 0.10	0.394 ± 0.025	0.217 ± 0.011	0.132 ± 0.005	0.184 ± 0.015
<i>k</i> _{cat} / <i>K</i> _M (s ⁻¹ mM ⁻¹)	17.8 ± 1.1	104 ± 5	331 ± 12	579 ± 16	414 ± 26
Δ(ΔG) (kJ mol ⁻¹)	-0.10	0.88	-0.42	0.32	1.28
					-0.14

^a Standard error^b Change of transition-state energy Δ(ΔG) = -RTln[(*k*_{cat}/*K*_M)_{mut} / (*k*_{cat}/*K*_M)_{wt}]

TABLE 8 (continued)

TABLE 9

Initial rates of glucose and isomaltose productions in the hydrolysis of 30% (w/v) maltodextrin M100 and 30% (w/v) glucose condensations, respectively, and their relative ratios for wild-type and mutant glucoamylases at 35°C, 45°C, and 55°C.

Enzymes	Initial rates		Ratios	
	Glucose ^a (G1) ($\mu\text{g}/\text{mL}\cdot\text{h}$) $\times 10^{-3}$	Isomaltose ^b (iG ₂) ($\mu\text{g}/\text{mL}\cdot\text{h}$) $\times 10^{-3}$	Ratios (iG ₂ /G1) $\times 10^6$	Relative ratios
35°C				
Wild-type	21.5 ± 0.6 ^c	289 ± 5	13.5	1.00
Lys108Arg	22.0 ± 0.4	969 ± 12	44.1	3.27
Tyr312Trp	17.9 ± 0.5	294 ± 4	16.4	1.21
311-314Loop	13.8 ± 0.4	128 ± 3	9.3	0.69
Ser30Pro/Gly137Ala	27.7 ± 0.4	298 ± 6	10.8	0.80
S-S/Ser30Pro/Gly137Ala	30.1 ± 0.5	245 ± 6	8.2	0.60
S-S	31.8 ± 0.6	135 ± 3	4.2	0.31
Ser436Pro	29.9 ± 0.6	903 ± 12	30.2	2.23
S-S/Ser436Pro	31.7 ± 0.5	824 ± 12	26.0	1.92
S-S/Gly137Ala	35.2 ± 0.6	982 ± 15	27.9	2.07
Gly137Ala/Ser436Pro	36.6 ± 0.7	776 ± 10	21.2	1.57
45°C				
Wild-type	66.2 ± 2.2	3880 ± 60	58.7	1.00
Lys108Arg	50.2 ± 2.0	6420 ± 110	128	2.18
Tyr312Trp	52.6 ± 2.1	3360 ± 60	63.9	1.09
311-314Loop	40.4 ± 1.8	1430 ± 40	35.3	0.60
Ser30Pro/Gly137Ala	76.3 ± 2.7	3690 ± 70	48.4	0.83
S-S/Ser30Pro/Gly137Ala	84.3 ± 3.0	3520 ± 60	41.7	0.71
S-S	86.3 ± 3.3	963 ± 28	11.2	0.19
55°C				
Wild-type	156 ± 3	4890 ± 80	31.3	1.00
Lys108Arg	101 ± 1	8200 ± 120	81.0	2.59
Tyr312Trp	110 ± 2	4440 ± 70	40.5	1.29

TABLE 9 (continued)

311-314Loop	128 ±2	1890 ± 50	14.8	0.47
Ser30Pro/Gly137Ala	157 ±3	7200 ± 110	45.8	1.47
S-S/Ser30Pro/Gly137Ala	167 ±3	5690 ± 100	34.1	1.09
S-S	164 ±3	1230 ± 40	7.5	0.24
Ser436Pro	218 ±3	4710 ± 80	21.6	0.69
S-S/Ser436Pro	ND ^d	5130 ± 100	ND	ND
SS/Gly137Ala	225 ±3	5720 ± 100	25.4	0.81
Gly137Ala/Ser436Pro	208 ±3	ND	ND	ND

^aSamples were taken from 30% (w/v) M100 hydrolysis reactions in 0.05 M NaOAc buffer, pH 4.4; glucose concentrations were determined by glucose oxidase method.

^bSamples were taken from 30% (w/v) glucose condensation reactions in 0.05 M NaOAc buffer, pH 4.4; isomaltose concentrations were determined by HPTLC.

^cStandard error

^dNot determined

Kinetic parameters of wild-type and mutant glucoamylase for hydrolysis of isomaltose and maltooligosaccharins of DP 2 - 7

Enzyme	Substrate					$\frac{k_{cat}/K_m (G2)}{k_{cat}/K_m (iG2)}$
	Isomaltose (iG2)	Maltose (G2)	Maltotriose (G3)	Maltotetraose (G4)	Maltopentaose (G5)	
Wild-type						
k_{cat} (S ⁻)	0.72 ± 0.01 ^b	20.4 ± 0.2	48.2 ± 0.7	64.5 ± 2.9	71.8 ± 1.9	73.7 ± 2.1
K_m (mM)	23.5 ± 0.6	1.01 ± 0.03	0.25 ± 0.014	0.111 ± 0.017	0.110 ± 0.010	0.107 ± 0.010
k_{cat}/K_m (S ⁻ mM ⁻¹)	0.031 ± 0.001	20.3 ± 0.55	19.6 ± 9	582 ± 65	65.4 ± 43	0.083 ± 0.004
Y48F/G9W						
k_{cat} (S ⁻)	ND ^c	9.9 ± 1.8	ND	ND	ND	ND
K_m (mM)	ND ^c	0.024 ± 0.003	ND	ND	ND	ND
k_{cat}/K_m (S ⁻ mM ⁻¹)	ND ^c	17.8	ND	ND	ND	ND
$\Delta(\Delta G)^c$ (kJ mol ⁻¹)	ND ^c	ND ^c	ND	ND	ND	ND
Y116W						
k_{cat} (S ⁻)	0.69 ± 0.02	11.7 ± 0.2	19.4 ± 0.3	50.9 ± 1.9	50.0 ± 1.7	53.1 ± 1.9
K_m (mM)	28.8 ± 2.5	0.98 ± 0.06	0.20 ± 0.01	0.20 ± 0.02	0.132 ± 0.014	0.143 ± 0.017
k_{cat}/K_m (S ⁻ mM ⁻¹)	0.024 ± 0.001	12.0 ± 0.60	98 ± 6	256 ± 17	378 ± 30	0.118 ± 0.008
$\Delta(\Delta G)$ (kJ mol ⁻¹)	0.67	1.39	1.84	2.17	1.45	1.62
Y175F						
k_{cat} (S ⁻)	1.02 ± 0.05	21.2 ± 0.2	40.0 ± 0.6	80.1 ± 1.8	79.6 ± 1.9	76.5 ± 1.5
K_m (mM)	40.1 ± 4.3	1.13 ± 0.04	0.29 ± 0.02	0.187 ± 0.012	0.120 ± 0.010	0.113 ± 0.008
k_{cat}/K_m (S ⁻ mM ⁻¹)	0.025 ± 0.002	18.8 ± 0.5	136 ± 6	429 ± 19	666 ± 42	0.095 ± 0.004
$\Delta(\Delta G)$ (kJ mol ⁻¹)	0.35	0.20	0.97	0.81	-0.05	0.03

^aDetermined at 45°C in 0.05 M sodium acetate buffer, pH 4.4.^bStandard error.^cChanges of transition-state energy $\Delta(\Delta G) = -RT \ln[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}]$.^dNot determined.

Enzyme	Isonicotinose (iG2)	Substrate						k_{cat}/K_m (G2)
		Maltose (G2)	Maltotriose (G3)	Maltotetraose (G4)	Maltopentaose (G5)	Maltohexaose (G6)	Maltoseptose (G7)	
R241K								261
k_{cat} (S ⁻¹)	1.34 ± 0.08 ^b	20.1 ± 0.3	46.8 ± 1.0	73.5 ± 7.2	70.7 ± 2.1	75.8 ± 2.7	80.6 ± 1.6	
K_m (mM)	39.3 ± 5.8	2.27 ± 0.11	0.62 ± 0.04	0.45 ± 0.09	0.19 ± 0.02	0.20 ± 0.02	0.20 ± 0.01	
k_{cat}/K_m (S ⁻¹ mM ⁻¹)	0.034 ± 0.003	8.9 ± 0.3	76 ± 3	164 ± 18	368 ± 21	373 ± 25	411 ± 16	
$\Delta(\Delta G)^c$ (kJ mol ⁻¹)	-0.28	2.19	2.51	3.36	1.52	1.61	1.98	
S411A								681
k_{cat} (S ⁻¹)	0.61 ± 0.02	18.9 ± 0.3	44.6 ± 0.1	58.5 ± 1.6	53.1 ± 1.2	54.7 ± 1.7	59.4 ± 0.6	
K_m (mM)	27.9 ± 2.9	1.26 ± 0.06	0.47 ± 0.04	0.182 ± 0.014	0.120 ± 0.009	0.115 ± 0.012	0.104 ± 0.004	
k_{cat}/K_m (S ⁻¹ mM ⁻¹)	0.022 ± 0.002	15.0 ± 0.5	94.1 ± 5.4	322 ± 18	413 ± 27	476 ± 41	570 ± 17	
$\Delta(\Delta G)$ (kJ mol ⁻¹)	0.84	0.80	1.94	1.56	1.15	0.96	1.12	
S411G								402
k_{cat} (S ⁻¹)	0.93 ± 0.06	23.0 ± 0.4	55.1 ± 1.6	59.7 ± 1.8	75.1 ± 2.1	75.9 ± 4.3	84.0 ± 2.5	
K_m (mM)	26.2 ± 2.7	1.59 ± 0.08	0.50 ± 0.04	0.092 ± 0.010	0.094 ± 0.010	0.125 ± 0.024	0.132 ± 0.012	
k_{cat}/K_m (S ⁻¹ mM ⁻¹)	0.036 ± 0.004	14.5 ± 0.6	108 ± 6	649 ± 55	795 ± 61	609 ± 87	634 ± 41	
$\Delta(\Delta G)$ (kJ mol ⁻¹)	-0.39	0.89	1.56	-0.29	-0.52	0.31	0.84	

^aDetermined at 45°C in 0.05 M sodium acetate buffer, pH 4.4.

^bStandard error.

^cChanges of transition-state energy $\Delta(\Delta G) = -RT \ln[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}]$.

^dNot determined.

TABLE 10 (continued)

Kinetic parameters of wild-type and mutant glucosaminylases for hydrolysis of isomaltose, maltose and maltoheptaose

Substrate	Wild-type	Mutant			
		S411G	S411A	S411C	S411H
Isomaltose (I2G)					
k_{cat} (S ⁻¹)	0.72 ± 0.01 ^b	0.93 ± 0.06	0.63 ± 0.02	0.22 ± 0.01	
K_m (mM)	23.5 ± 0.6	26.2 ± 2.7	27.9 ± 2.9	12.3 ± 0.9	ND ^d
k_{cat}/K_m (S ⁻¹ mM ⁻¹)	0.031 ± 0.001	0.036 ± 0.004	0.022 ± 0.002	0.018 ± 0.001	
$\Delta(\Delta G)^e$ (kJ mol ⁻¹)	-	-0.39	0.84	1.4	
Maltose (G2)					
k_{cat} (S ⁻¹)	20.4 ± 0.2	23.0 ± 0.4	18.9 ± 0.3	7.78 ± 0.07	5.31 ± 0.15
K_m (mM)	1.01 ± 0.03	1.59 ± 0.08	1.26 ± 0.06	0.53 ± 0.02	3.67 ± 0.25
k_{cat}/K_m (S ⁻¹ mM ⁻¹)	20.3 ± 0.6	14.5 ± 0.6	15.0 ± 0.5	14.8 ± 0.6	1.45 ± 0.06
$\Delta(\Delta G)^e$ (kJ mol ⁻¹)	-	0.89	0.80	0.83	6.98
Maltoheptaose (G7)					
k_{cat} (S ⁻¹)	72.3 ± 0.9	84.0 ± 2.5	59.4 ± 0.6	33.0 ± 0.5	32.4 ± 0.9
K_m (mM)	0.083 ± 0.004	0.132 ± 0.012	0.104 ± 0.004	0.070 ± 0.005	0.336 ± 0.024
k_{cat}/K_m (S ⁻¹ mM ⁻¹)	870 ± 35	634 ± 41	570 ± 17	474 ± 25	97 ± 5
$\Delta(\Delta G)^e$ (kJ mol ⁻¹)	-	0.84	1.12	1.60	5.81

^aDetermined at 45°C in 0.05 M sodium acetate buffer, pH 4.4.^bStandard error.^cChanges of transition-state energy $\Delta(\Delta G) = -RT \ln[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}]$.^dNot determined.

pK values and optimum pH of wild-type and mutant glucosidases for hydrolysis of maltose and maltoheptaose at 45°C

	Free enzyme (uncomplexed)				Enzyme-substrate complex (maltose-complexed)				Enzyme-substrate complex (maltoheptaose-complexed)			
	pK ₁	pK ₂	pH _{opt}	pK ₁	pK ₂	pH _{opt}	pK ₁	pK ₂	pH _{opt}	pK ₁	pK ₂	pH _{opt}
Wild-type	2.77	5.80	4.29	2.11	5.85	3.98	2.60	6.78	4.69			
S411G	3.01	5.57	4.29	2.68	5.81	4.24	3.22	6.73	4.98			
S411A	3.11	5.86	4.49	3.32	6.32	4.82	3.91	7.18	5.54			
S411C	3.26	5.86	4.56	3.32	6.38	4.85	ND*	ND	ND			
S411H	3.10	5.01	4.05	3.58	4.69	4.13	ND	ND	ND			
S411D	3.13	5.72	4.42	3.34	5.53	4.44	ND	ND	ND			

*Not determined.

TABLE 13

Increases in free energies for thermal inactivation ($\Delta\Delta G^\ddagger$) relative to wild-type GA calculated at 65°C

GA form	$\Delta\Delta G^\ddagger$ (kJ/mol)
S436P	0.5
S30P	1.6
G137A	0.8
S-S	1.2
S-S/S436P	2.2
G137A/S436P	2.0
S30P/G137A	4.5
S-S/S30P	3.5
S-S/G137A	2.7
S-S/S30P/G137A	4.4

^a $\Delta\Delta G^\ddagger$ greater than zero indicates increased thermostability

TABLE 14

Decrease in the relative ratio of initial rate of isomaltose formation from 30% (w/v) glucose condensation reactions to that of glucose formation in 30% (w/v) maltodextrin M100 hydrolysis reactions.

GA form	Relative ratios ^a
Wild-type	1.00
S-S	0.24
S30P	0.77
G137A	0.54
Y175F	0.76
300Loop	0.47
S411A	0.40
S411G	0.38
S436P	0.70
S-S/G137A	0.81
G121A/S411G	0.44

All the above reactions were carried out in 0.05M sodium acetate buffer, pH4.4, at 55°C.

^aRatios lower than 1.00 indicate increased specificity for α -(1,4) over α -(1,6)-linked substrates.

TABLE 15

Increase in the optimum pH of the enzyme-substrate complex of mutant glucoamylases for hydrolysis of maltose at 45°C compared to that of wild-type.

GA form	pH _{opt} Increase*
S411G	0.26
S411A	0.84
S411C	0.86
S411H	0.15
S411D	0.46

*The pH optimum of the enzyme-substrate complex of wildtype glucoamylase for hydrolysis of maltose at 45°C was pH 3.98.

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SEQUENCE LISTING

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- (i) APPLICANT: Allen, Martin
Fang, Tsuei-Yun
Li, Yuxing
Liu, Hsuan-Liang
Chen, Hsui-Mei
Coutinho, Pedro
Hanzatko, Richard
Ford, Clark
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THERMOSTABILITY
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 - (E) COUNTRY: US
 - (F) ZIP: 48334
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Kohn, Kenneth I.
 - (B) REGISTRATION NUMBER: 30,955
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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 616 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Aspergillus

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ala Thr Leu Asp Ser Trp Leu Ser Asn Glu Ala Thr Val Ala Arg Thr
1 5 10 15

Ala Ile Leu Asn Asn Ile Gly Ala Asp Gly Ala Trp Val Ser Gly Ala
20 25 30

Asp Ser Gly Ile Val Val Ala Ser Pro Ser Thr Asp Asn Pro Asp Tyr
35 40 45

Phe Tyr Thr Trp Thr Arg Asp Ser Gly Leu Val Leu Lys Thr Leu Val
50 55 60

Asp Leu Phe Arg Asn Gly Asp Thr Ser Leu Leu Ser Thr Ile Glu Asn
65 70 75 80

Tyr Ile Ser Ala Gln Ala Ile Val Gln Gly Ile Ser Asn Pro Ser Gly
85 90 95

Asp Leu Ser Ser Gly Ala Gly Leu Gly Glu Pro Lys Phe Asn Val Asp
100 105 110

Glu Thr Ala Tyr Thr Gly Ser Trp Gly Arg Pro Gln Arg Asp Gly Pro
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Ala Leu Arg Ala Thr Ala Met Ile Gly Phe Gly Gln Trp Leu Leu Asp
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Asn Gly Tyr Thr Ser Thr Ala Thr Asp Ile Val Trp Pro Leu Val Arg
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Asn Asp Leu Ser Tyr Val Ala Gln Tyr Trp Asn Gln Thr Gly Tyr Asp
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Leu Trp Glu Glu Val Asn Gly Ser Ser Phe Phe Thr Ile Ala Val Gln
180 185 190

His Arg Ala Leu Val Glu Gly Ser Ala Phe Ala Thr Ala Val Gly Ser
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Ser Cys Ser Trp Cys Asp Ser Gln Ala Pro Glu Ile Leu Cys Tyr Leu
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Gln Ser Phe Trp Thr Gly Ser Phe Ile Leu Ala Asn Phe Asp Ser Ser
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Arg Ser Gly Lys Asp Ala Asn Thr Leu Leu Gly Ser Ile His Thr Phe
245 250 255

Asp Pro Glu Ala Ala Cys Asp Asp Ser Thr Phe Gln Pro Cys Ser Pro
260 265 270

Arg Ala Leu Ala Asn His Lys Glu Val Val Asp Ser Phe Arg Ser Ile
275 280 285

Tyr Thr Leu Asn Asp Gly Leu Ser Asp Ser Glu Ala Val Ala Val Gly
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Arg Tyr Pro Glu Asp Thr Tyr Tyr Asn Gly Asn Pro Trp Phe Leu Cys
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Thr Leu Ala Ala Ala Glu Gln Leu Tyr Asp Ala Leu Tyr Gln Trp Asp
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Lys Gln Gly Ser Leu Glu Val Thr Asp Val Ser Leu Asp Phe Phe Lys
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Ala Leu Tyr Ser Asp Ala Ala Thr Gly Thr Tyr Ser Ser Ser Ser
 355 360 365

Thr Tyr Ser Ser Ile Val Asp Ala Val Lys Thr Phe Ala Asp Gly Phe
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Val Ser Ile Val Glu Thr His Ala Ala Ser Asn Gly Ser Met Ser Glu
 385 390 395 400

Gln Tyr Asp Lys Ser Asp Gly Glu Gln Leu Ser Ala Arg Asp Leu Thr
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Trp Ser Tyr Ala Ala Leu Leu Thr Ala Asn Asn Arg Arg Asn Ser Val
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Val Pro Ala Ser Trp Gly Glu Thr Ser Ala Ser Ser Val Pro Gly Thr
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Cys Ala Ala Thr Ser Ala Ile Gly Thr Tyr Ser Ser Val Thr Val Thr
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Ser Trp Pro Ser Ile Val Ala Thr Gly Gly Thr Thr Thr Ala Thr
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Pro Thr Gly Ser Gly Ser Val Thr Ser Thr Ser Lys Thr Thr Ala Thr
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Ala Ser Lys Thr Ser Thr Ser Thr Ser Ser Cys Thr Thr Pro
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Thr Ala Val Ala Val Thr Phe Asp Leu Thr Ala Thr Thr Thr Tyr Gly
 515 520 525

Glu Asn Ile Tyr Leu Val Gly Ser Ile Ser Gln Leu Gly Asp Trp Glu
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Thr Ser Asp Gly Ile Ala Leu Ser Ala Asp Lys Tyr Thr Ser Ser Asp
 545 550 555 560

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 565 570 575

Tyr Lys Phe Ile Arg Ile Glu Ser Asp Asp Ser Val Glu Trp Glu Ser
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Asp Pro Asn Arg Glu Tyr Thr Val Pro Gln Ala Cys Gly Thr Ser Thr
 595 600 605

Ala Thr Val Thr Asp Thr Trp Arg
 610 615

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 7 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide

- 78 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Asn Gly Asn Gly Asn Ser Gln
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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31

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AAAGTCCAGCG ACACAGGTGT GACCTCCAAC GAC

33

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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32

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid

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- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

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32

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(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATCGGGGCGG ACGGTTGTTG GGTGTCGGGC GCG

33

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

-80-

GGTCTCGGTG AGCCCAGGTT CAATGTCGAT

30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "PRIMER"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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30

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "PRIMER"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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27

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "PRIMER"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TACCCCTGAGG ACACGTACAA CGGCAACGGC AACTCGCAGG GCAACCCGTG GTTCCTGTGC

60

CLAIMS

What is claimed is:

1. A fungal glucoamylase including a mutation pair Asn20Cys coupled with Ala27Cys forming a disulfide bond between the two members of the pair.
2. The glucoamylase as set forth in claim 1 wherein the mutation provides increased thermal stability and reduced isomaltose formation.
3. The fungal glucoamylase as set forth in claim 1 and including at least one mutations selected from Table 13 wherein cumulative thermal stability is provided by the additional mutations.
4. The fungal glucoamylase as set forth in claim 1 further including mutations Ser30Pro, Gly137Ala wherein cumulative thermal stability is provided by the additional mutations.
5. The fungal glucoamylase as set forth in claim 1 and including at least one mutation from Table 14 wherein cumulative reduced isomaltose formation is provided by the additional mutations.
6. The fungal glucoamylase as set forth in claim 1 further including a 311-314Loop mutation wherein cumulative reduced isomaltose formation is provided by the mutation.
7. A fungal glucoamylase including a 311-314Loop mutation.
8. The glucoamylase as set forth in claim 7

wherein reduced isomaltose formation is provided by the mutation.

9. The fungal glucoamylase as set forth in claim 7 and including at least one mutation from Table 14 wherein cumulative reduced isomaltose formation is provided by the additional mutation.

10. A fungal glucoamylase including a mutation Ser411Ala.

11. The glucoamylase as set forth in claim 10 wherein increased pH optimum and reduced isomaltose formation is provided by the mutation.

12. The fungal glucoamylase as set forth in claim 10 and including at least one mutation from Table 15 wherein cumulative increased pH optimum is provided by the mutations.

13. The fungal glucoamylase as set forth in claim 10 and including at least one mutation from Table 14 wherein cumulative reduced isomaltose formation is provided by the mutations.

14. A fungal glucoamylase including a Ser411Ala mutation and a mutation pair Asn20Cys coupled with Ala27Cys forming a disulfide bond between the two members of the pair.

15. The glucoamylase as set forth in claim 14 wherein increased thermal stability, increased pH optimum and reduced isomaltose formation are provided by the mutations.

16. A fungal glucoamylase including a Ser411Ala

mutation and a mutation pair Asn20Cys coupled with Ala27Cys forming a disulfide bond between the two members of the pair and a 311-314Loop mutation.

17. The glucoamylase as set forth in claim 16 wherein increased thermal stability, increased pH optimum and reduced isomaltose formation are provided by the mutations.

18. A vector containing the cDNA for an engineered glucoamylase as set forth in claims 1-17.

19. A host cell transformed with the vector of claim 18.

20. A fungal glucoamylase as set forth in claims 1-17 wherein the glucoamylase is an *Aspergillus* glucoamylase.

21. The glucoamylase as set forth in claim 20 wherein the glucoamylase is *Aspergillus awamori* glucoamylase.

22. A method to obtain a fungal glucoamylase with decreased thermal inactivation by designing mutations having decreased conformational entropy of unfolding, or increased stability of α -helices, or increased disulfide bonds or hydrogen bonding and electrostatic interactions and hydrophobic interactions and Vanderwalls interactions and packing compactness.

23. A method to obtain a fungal glucoamylase with reduced isomaltose formation by designing mutations to decrease the α -(1,6)-glucosidic linkage affinity.

24. A method to obtain a fungal glucoamylase with

increased pH optimum by changing the polarity, charge distribution and hydrogen bonding in the microenvironment of the catalytic base Glu400.

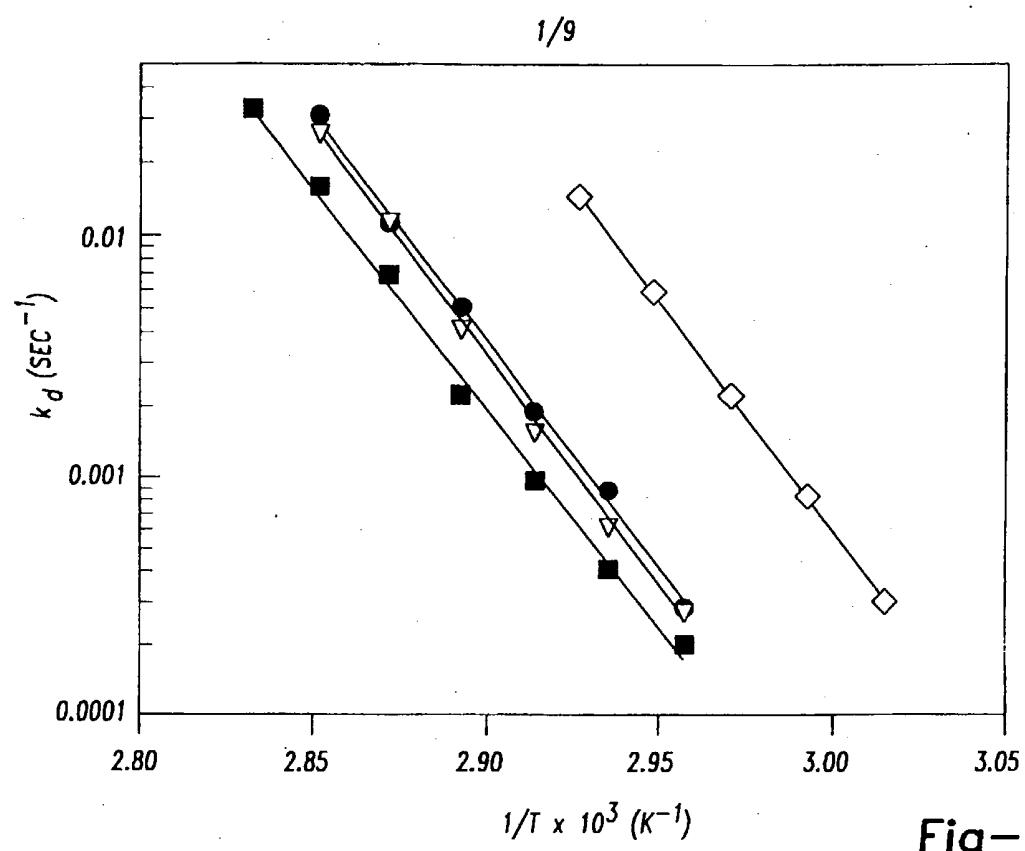
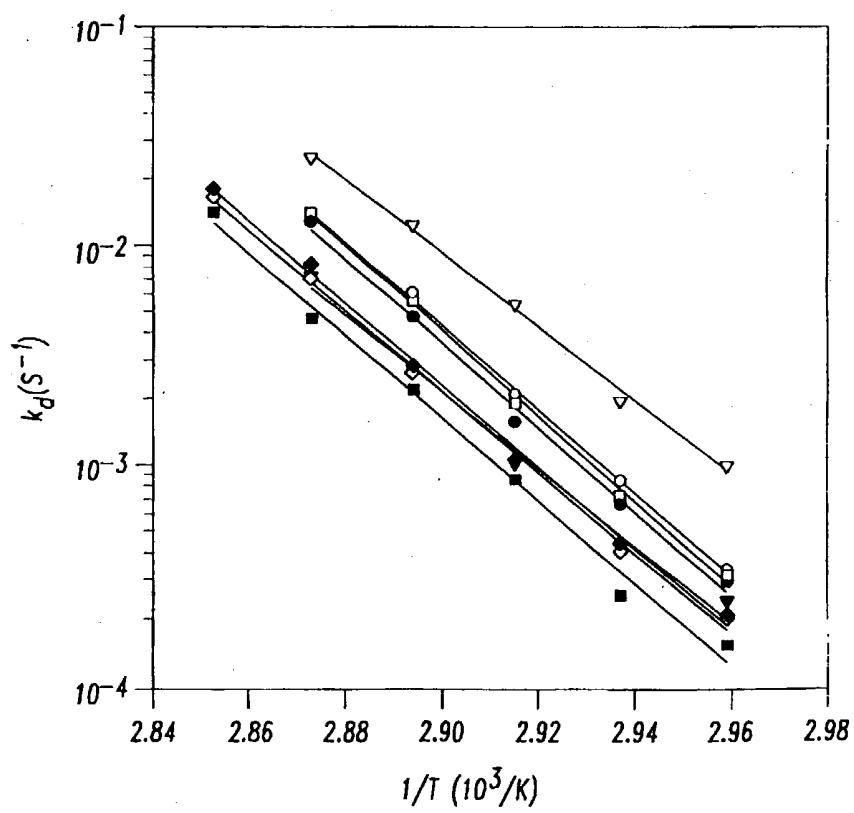
25. A method of selecting mutations for fungal glucoamylase to be used for constructing glucoamylases with cumulative mutations by

designing and generating individual mutations by site directed mutagenesis;

screening the individual mutations and selecting those which show at least increased pH optimum, decreased irreversible thermal inactivation rates or reduced isomaltose formation;

performing site directed mutagenesis to produce enzymes carrying at least two of the isolated selected mutations for either increased pH optimum, decreased irreversible thermal inactivation rates or reduced isomaltose formation; and

screening for cumulatively additive effects of the mutations on pH optimum, thermal stabilizing or reduced isomaltose formation by the produced enzymes carrying at least two of the isolated selected mutations.

Fig-1Fig-2

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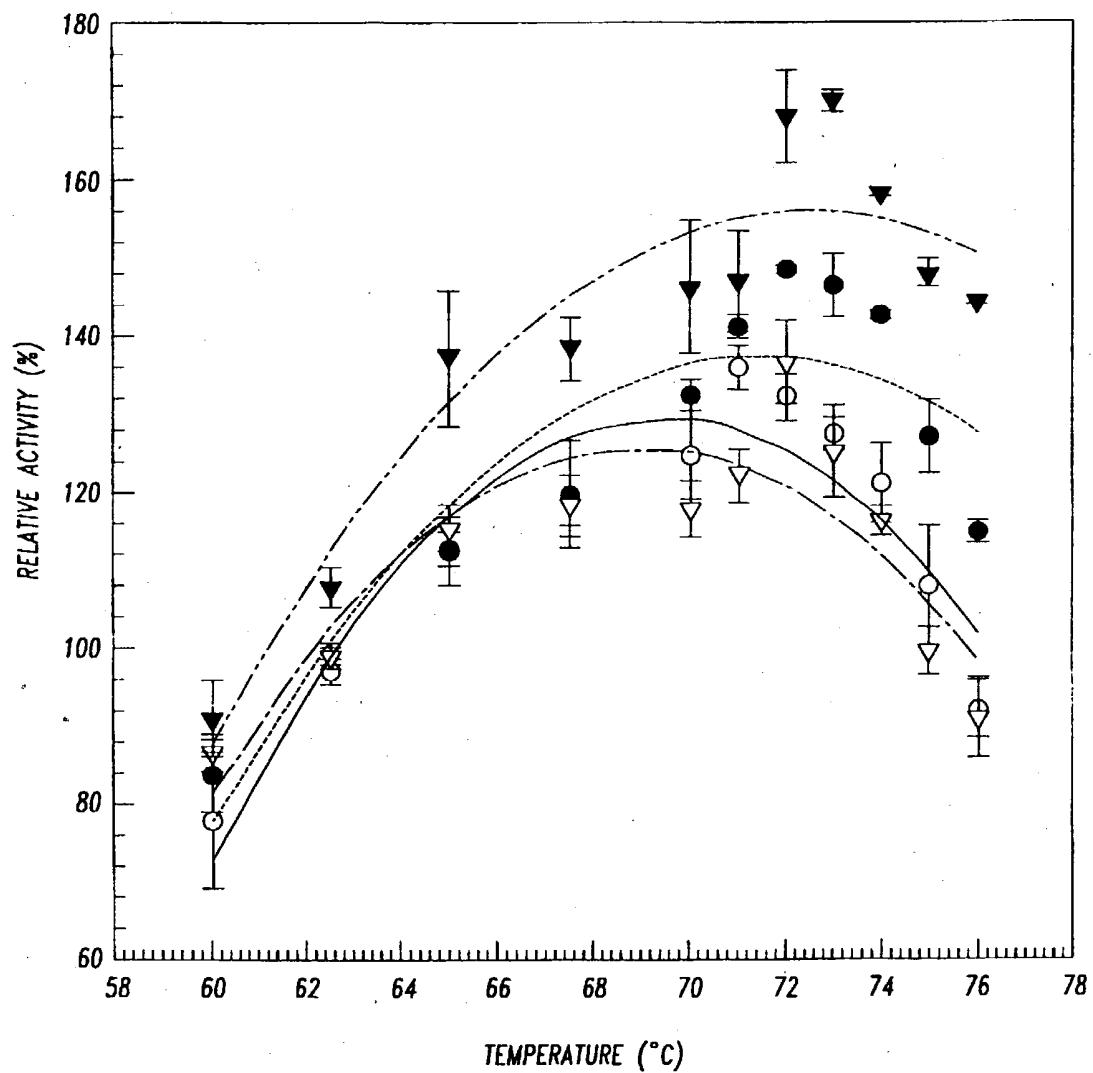
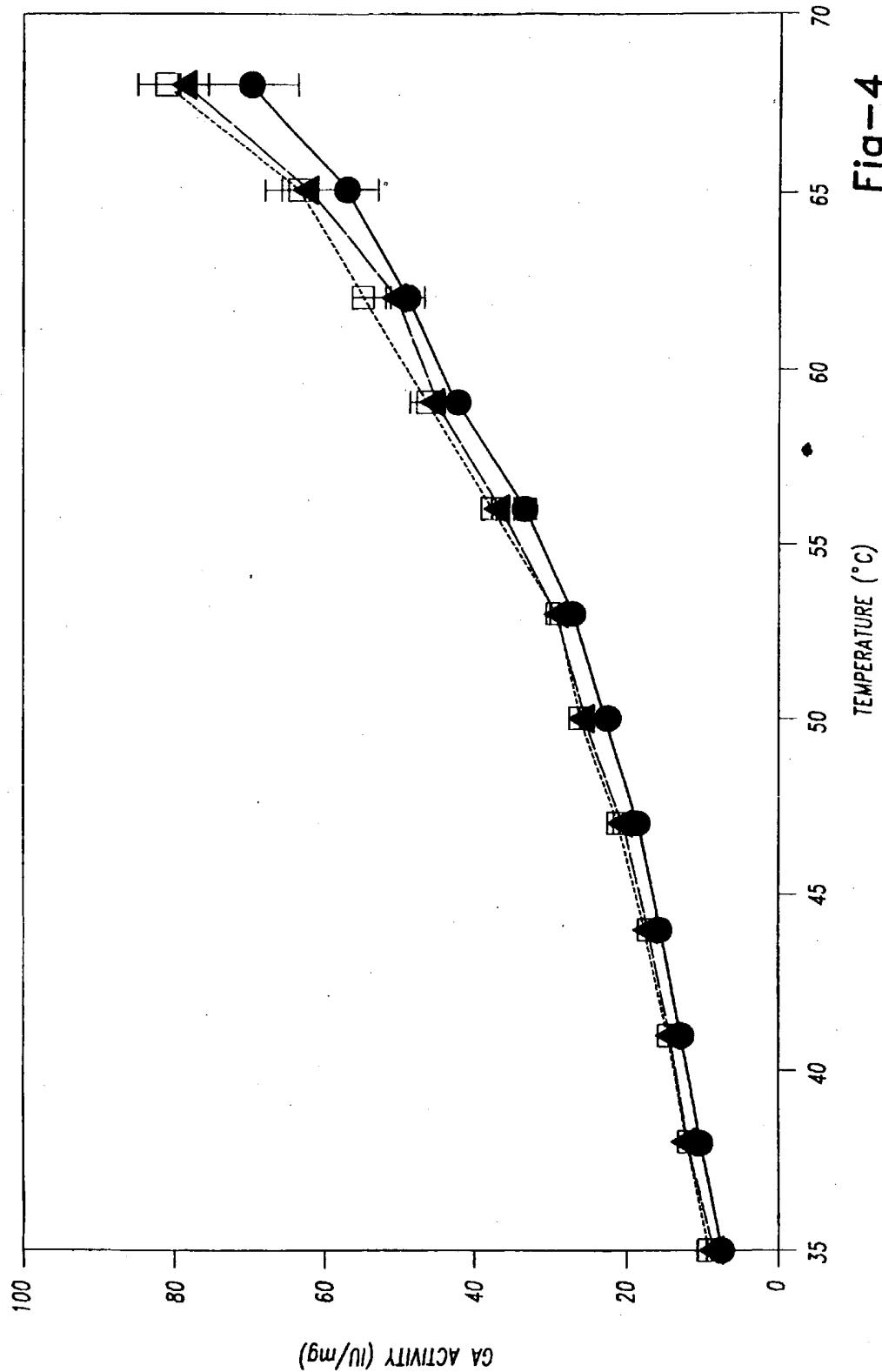


Fig-3

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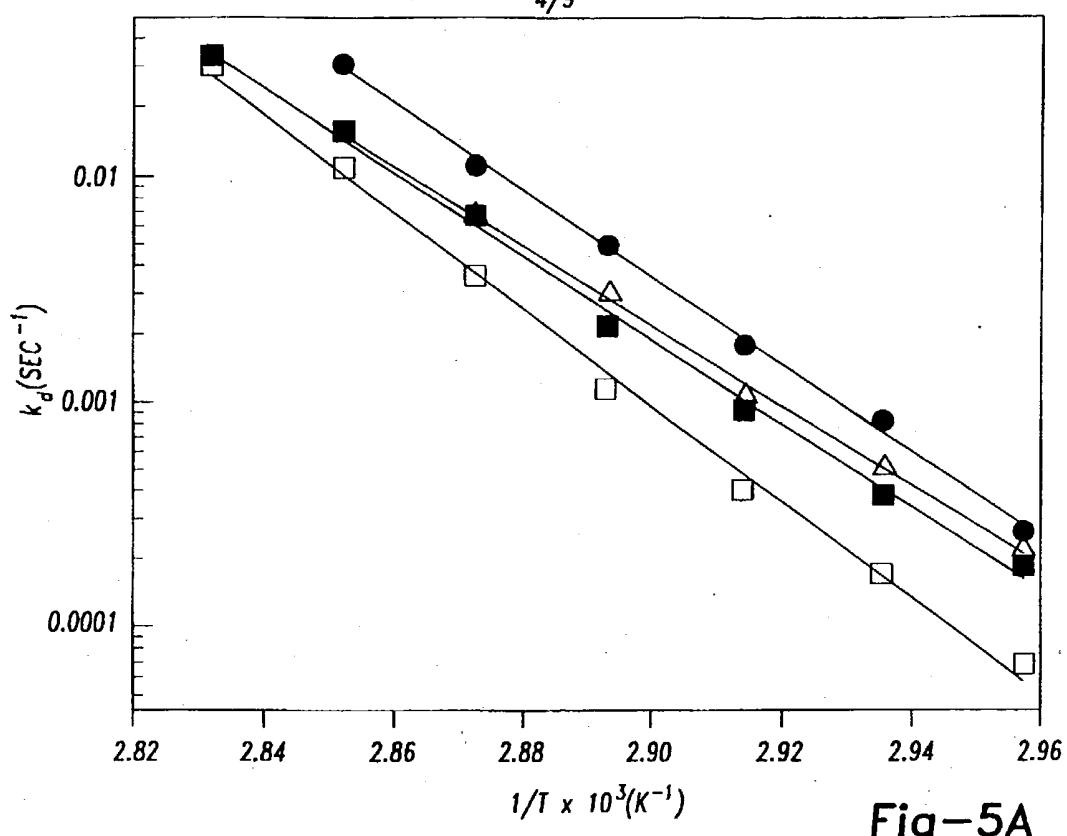


Fig-5A

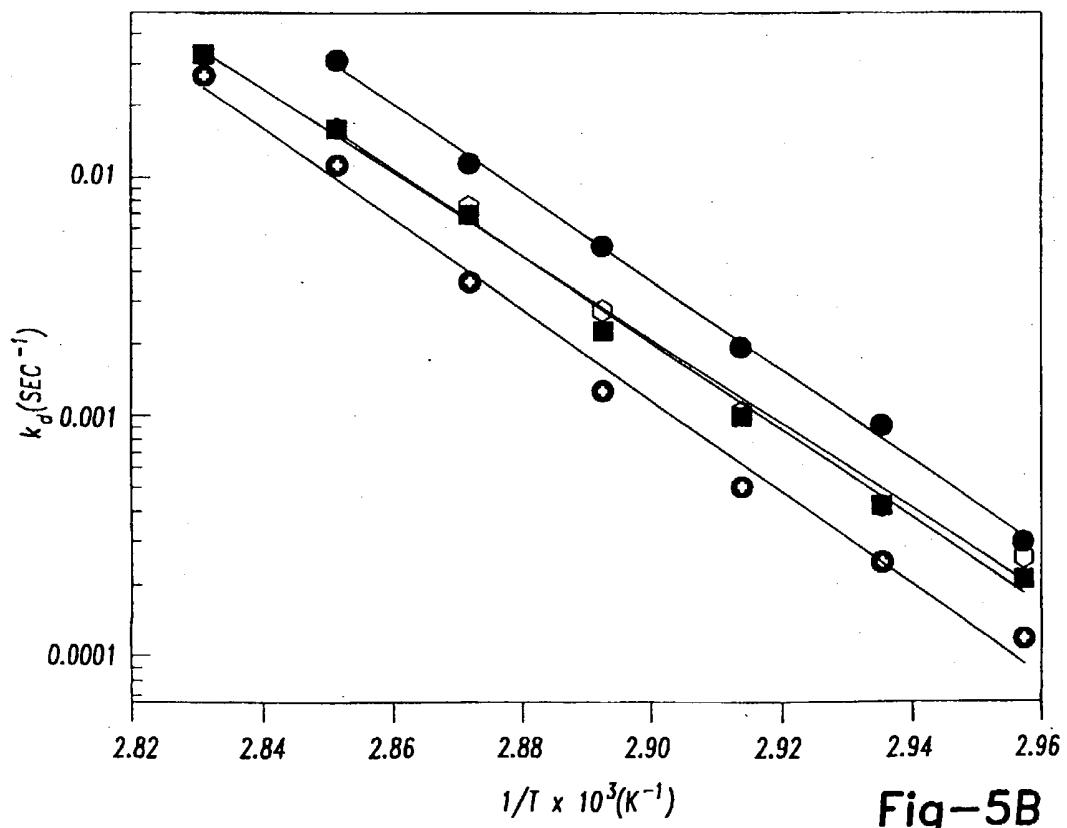


Fig-5B

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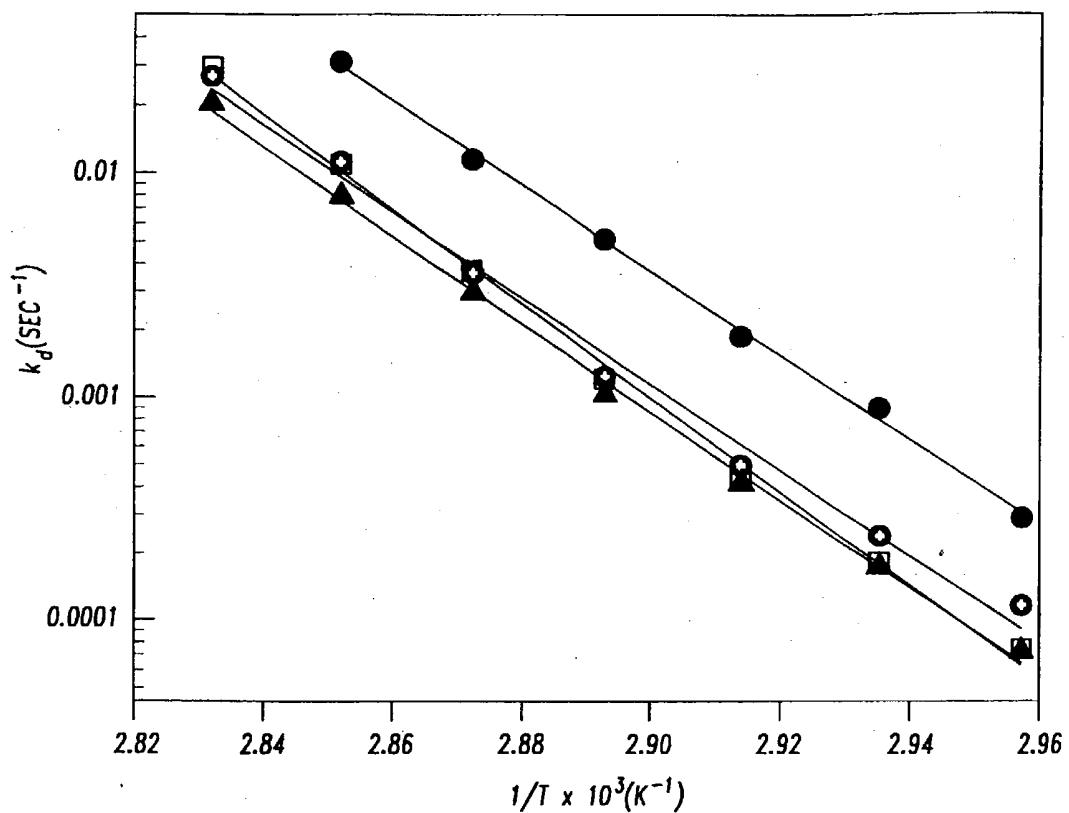


Fig-5C

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55 °C

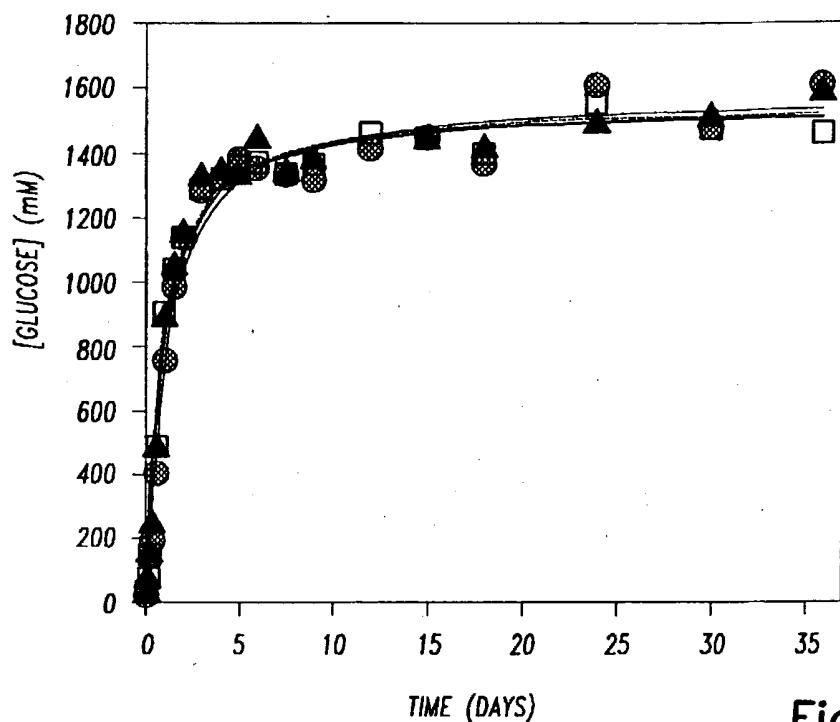


Fig-6A

65 °C

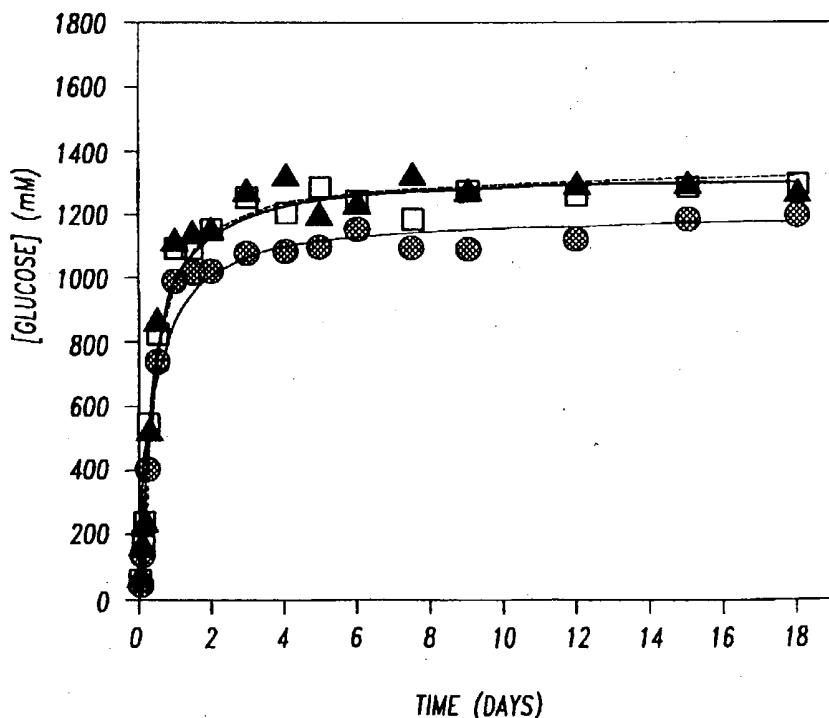
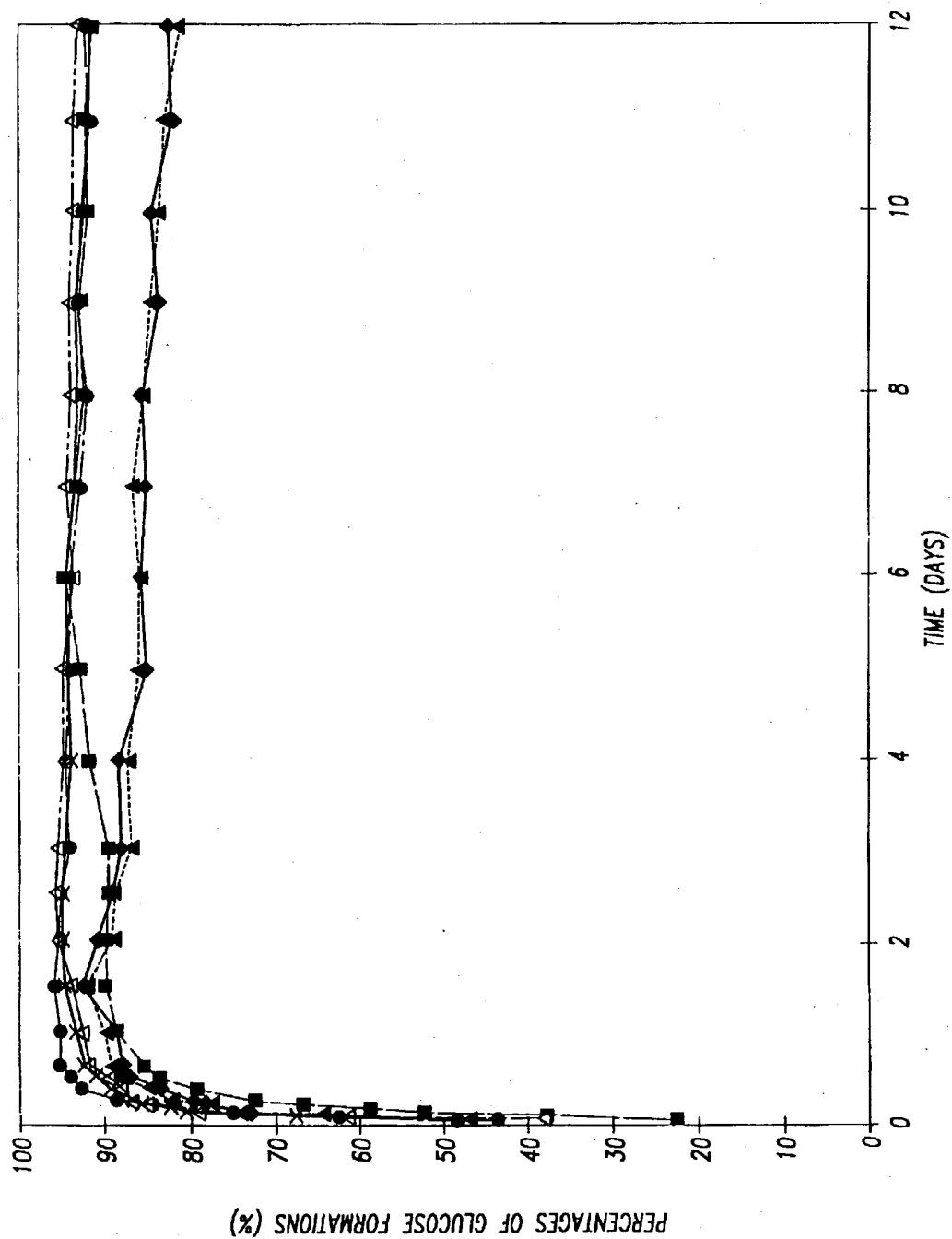


Fig-6B

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Fig-7

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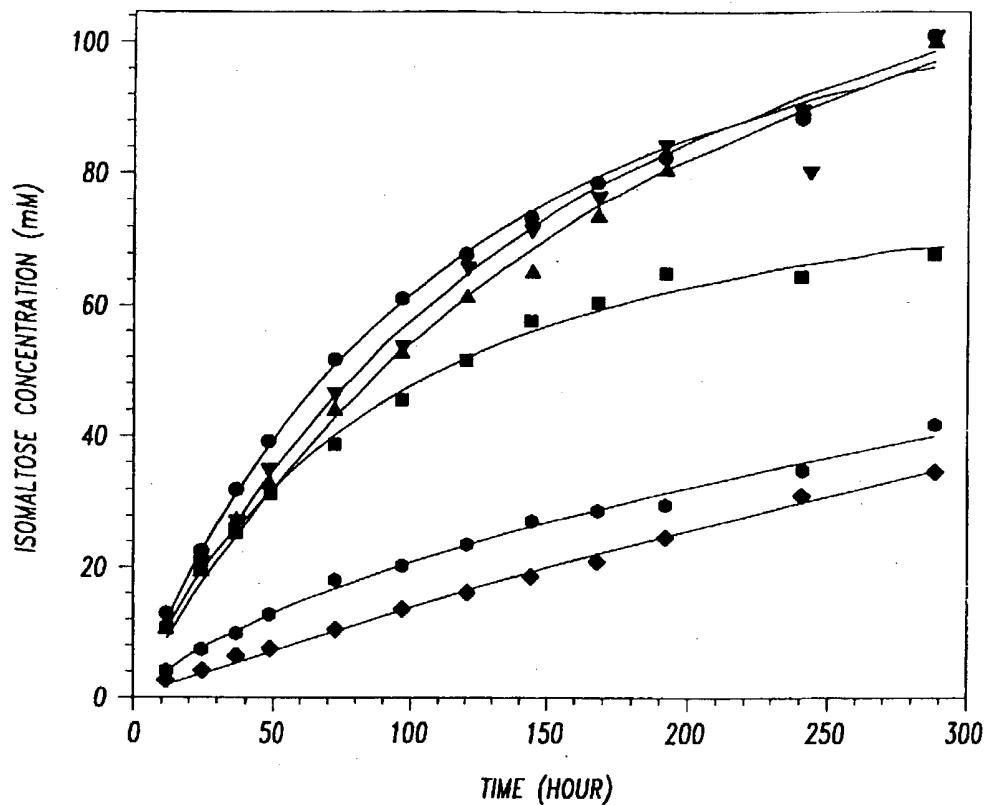


Fig-8

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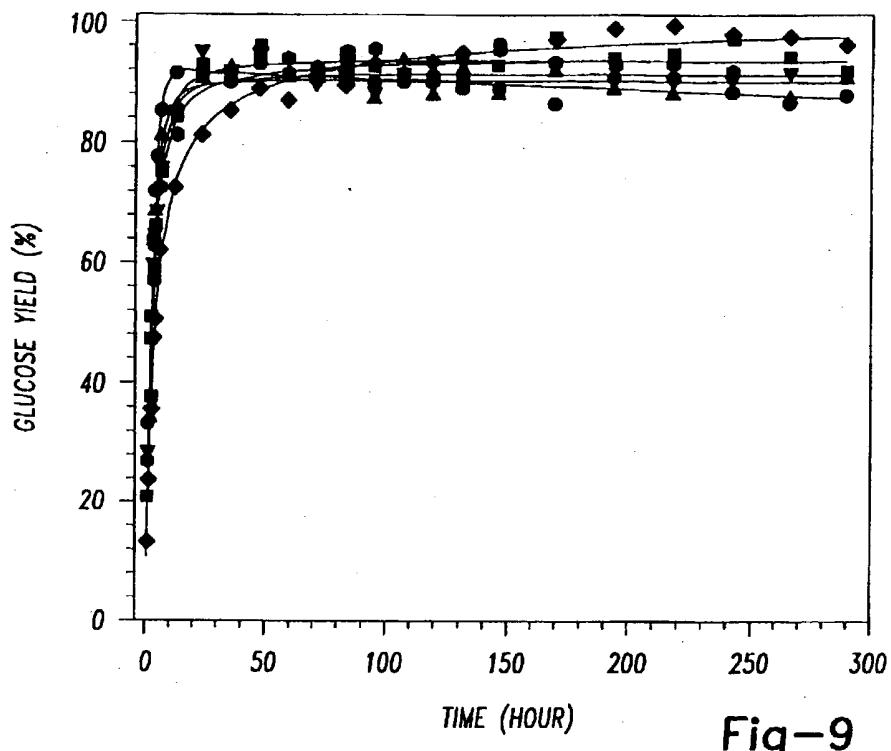


Fig-9

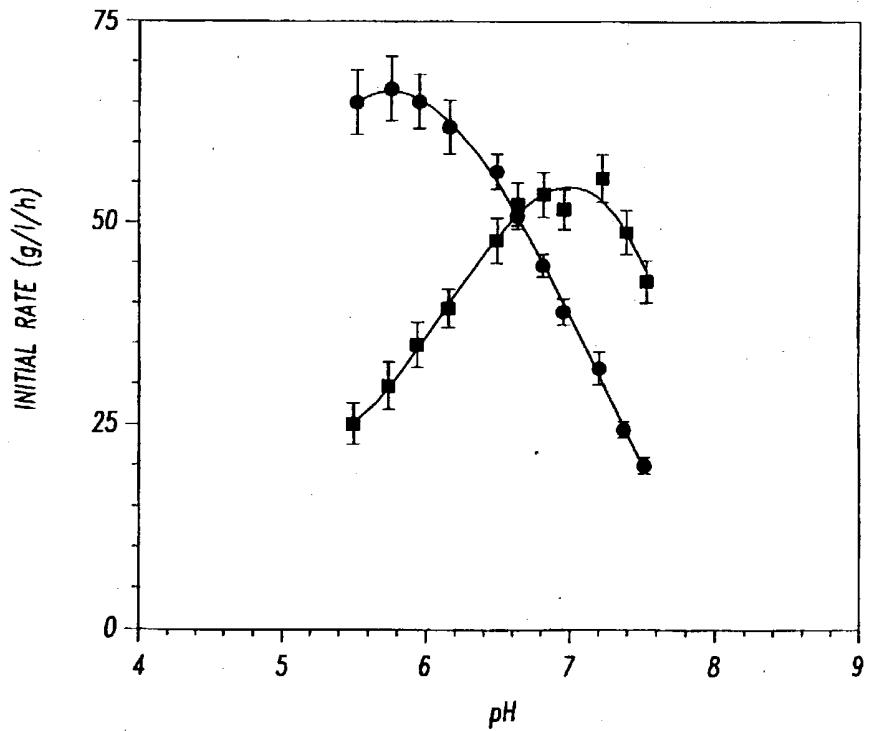


Fig-10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/12983

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) C12N 9/34, 1/21, 15/63, 15/09

US CL 435/205, 252.3, 320.1, 172.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/205, 252.3, 320.1, 172.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P ---	Li. Genetic construction and biochemical analysis of thermostability mutants of glucoamylase from aspergillus awamori. Dissertation Abstracts International. Abstract No. 97:24090, 1996, Vol. 57, No. 11B, page 6761, see abstract.	1, 2, 22 -----
Y, P ---	CHEN. Site-directed mutagenesis to enhance thermostability of Aspergillus awamori glucoamylase expressed in Saccharomyces cerevisiae. Dissertation Abstracts International. Abstract No. 95(03):B0023, 1994, Vol. 54, page 5998, see abstract.	3-6, 25 -----
X ---		22 -----
Y		3-5, 9, 13

 Further documents are listed in the continuation of Box C. See patent family annex.

•	Special categories of cited documents:	
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"B"	earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"A" document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

30 SEPTEMBER 1997

Date of mailing of the international search report

31 OCT 1997

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Telephone No. (703) 308-0196

Facsimile No. (703) 305-3230

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/12983

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEN et al. Effect of replacing helical glycine residues with alanines on reversible and irreversible stability and production of <i>Aspergillus awamori</i> glucoamylase. <i>Protein Engineering</i> . June 1996, Vol. 9, No. 6, pages 499-505, see whole document.	22
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Y		3-5, 9, 13
Y,P	STOFFER et al. Glucoamylase mutants in the conserved active-site segment Trp170-Tyr175 located at a distance from the site of catalysis. <i>Protein Engineering</i> . January 1997, Vol. 10, No. 1, pages 81-87, see whole document.	5, 9, 13
X	FIEROBE et al. Mutational modulation of substrate bond-type specificity and thermostability of glucoamylase from <i>Aspergillus awamori</i> by replacement with short homologue active site sequences and thiol/disulfide engineering. <i>Biochemistry</i> . 02 July 1996, Vol. 35, No. 26, pages 8696-8704, see whole document.	22
---		---
Y		25
X	SIERCKS et al. Protein engineering of the relative specificity of glucoamylase from <i>Aspergillus awamori</i> based on sequence similarities between starch-degrading enzymes. <i>Protein Engineering</i> . December 1994, Vol. 7, No. 12, pages 1479-1484, see whole document.	23
---		---
Y		25
X	BAKIR et al. Cassette mutagenesis of the active site of <i>Aspergillus awamori</i> glucoamylase. <i>Protein Engineering</i> . Abstract No. 93-04739, March 1993, Vol. 6, Suppl., page 41, see abstract.	24
---		---
Y		25

INTERNATIONAL SEARCH REPORT

International application No. PCT/US97/12983

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

STN-USpatfull, CJACS, Europatfull, Biotechds, CAPIus, CJElsevier, Medline, Biosis, Scisearch, Toxline, JICST-EPlus, Embase, FSTA, Agricola, Cancerlit, Hipat, CEABA, Lifesci, Toxlit, Prompt, CABA, Dissabs, WPIDS, Biobusiness, Paperchem2, Aquasci, CEN, Japio, Patosep
search terms: glucoamylase, mutant, mutation, mutagenesis, variant, modify, alter, homolog, analog, mutein, substitute, thermostability, isomaltose, pH optimum